國立臺灣大學生命科學院動物學研究所

碩士論文

Graduate Institute of Zoology
College of Life Science
National Taiwan University

Master Thesis

前腦室側視丘對機械性痛敏感症的重要性

Anterior Nucleus of Paraventricular Thalamus is Important in Mechanical Hyperalgesia

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中華民國 100 年元月

January, 2011

國立臺灣大學碩士學位論文
口試委員會審定書

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本論文係張雅婷（R97b41039）在國立臺灣大學生命科學院動物學研究所完成之碩士學位論文，於民國一百年元月十七日承下列考試委員審查通過及口試及格，特此證明

口試委員：閔明源（指導教授）

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ACKNOWLDEGEMENT

I owe my deepest gratitude to both of my advisors Dr Chen Chien-chang and Dr Min Ming-Yuan in fully support my study. Especially Dr Chen, who has the greatest passion in life and science, whose warm encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of this project and the attitude of Science.

I am heartily thankful to my committee, Dr Chen Chih-Cheng for the kindly and practical suggestions and advices in working scientifically and thinking both delicately and broadly about this issue. It is also my greatest pleasure to thank those who I ever collaborated with. I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

It is my honor to praise my earnest gratitude to those who accomplished me to walk through all the years from putting away the teacher's guide and picking up textbooks and reconnecting my scientific nerves, my family and friends. Particular to Mel, Janette, Hitachi and Whats, this thesis would not be possibly to start without your most heartily encouragements.

To dad and mum and my dearest friends, without you, I won't be here.

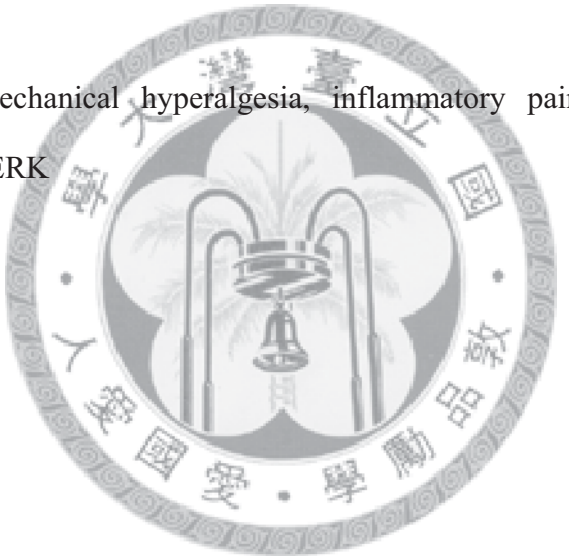
CONTENTS

I. Abstract

Central sensitization is important for development and maintenance of mechanical hyperalgesia in inflammatory, neuropathic and chronic widespread pain. The supraspinal mechanism for central sensitization is not completely understood in most pain models. Enhanced pain-related synaptic transmission from parabrachial area (PB) to central nucleus of amygdala (CeA) has been demonstrated in inflammatory and neuropathic pain. It has been shown that manipulation of ERK activity in CeA can alter the mechanical hyperalgesia induced by formalin in mice. We have recently shown that acid-induced chronic mechanical hyperalgesia also induced ERK activation in CeA and in the anterior nucleus of paraventricular thalamus (PVA) in mice. Blocking ERK activity in PVA blunts the chronic mechanical hyperalgesia. We therefore hypothesize ERK activation in PVA contributes to mechanical hyperalgesia in different pain models. To test this hypothesis, we examined the ERK activity in PVA in formalin-induced inflammatory pain and spared nerve injury (SNI)-induced neuropathic pain models and determined the association between ERK activation and mechanical hyperalgesia in female C57B6 mice. Our results showed that pERK staining in PVA were enhanced in both formalin- and SNI- induced mechanical hyperalgesia compared to their control. Manipulation ERK activity in PVA alters formalin-induced, SNI-induced and acid-induced persistent mechanical hyperalgesia. The direct infusion of PDBu in PVA induced mechanical but not thermal hyperalgesia in naïve mice further pointed out the

importance of PVA in nociception transmission in brain circuit. Finally, the repeated infusion of PDBu in PVA induced the persistent mechanical hyperalgesia strongly support that ERK activation in PVA plays a crucial role in modulation of mechanical hyperalgesia. The discovery of the importance of PVA in the central mechanism of nociceptive sensation is attributable not only to the basic understanding of pain perception but also to patients who are suffered from pain issues.

Keywords: PVA, mechanical hyperalgesia, inflammatory pain, neuropathic pain, central sensitization, ERK



I. 摘要

中樞神經敏感化（central sensitization）對於發炎性（inflammatory pain），神經病理性（neuropathic pain）及慢性廣泛性疼痛（chronic widespread pain）所誘發之機械性痛敏感症（mechanical hyperalgesia）具有重要的生理調節的意義，然而在腦部這個層次中，我們認知的中樞神經敏感化對痛覺調控功能之瞭解是微渺不足的。學者們在神經電生理的研究指出，發炎性及神經病理性疼痛，會誘發由亞臂核（parabrachial area）到杏仁核中間核區（central nucleus of amygdala）突觸傳導增加的現象；加上在動物行為及生理的研究中，在承受以福馬林測試誘發發炎性疼痛的小鼠身上，也發現了改變杏仁核中間核區的胞外信號調節激素(ERK)的生理活性，能調控機械性痛敏症之增緩。

我們進期的研究更指出，受到酸刺激引發慢性肌肉痛的小鼠的杏仁核中間核區及前腦室側視丘（anterior nucleus of paraventricular thalamus，PVA）中之磷酸化胞外信號調節激素 (pERK) 會被大量的活化，抑制在前腦室側視丘之胞外信號調節激素的活化反應，可進而絕斷慢性痛敏感性的發展。因此，我們提出調節胞外信號調節激素在前腦室側視丘的活性可以調控眾誘因造成之機械性痛敏感症之假說。

我們採用福馬林測試（formalin test）及坐骨神經分支選擇結紮切斷模式（spared nerve injury model, SNI）二種痛覺模式。首先，我們檢測以不同小鼠痛覺模式誘發機械性疼痛的小鼠的前腦室側視丘之調節胞外信號調節激素的活性；進而，我們鑑定胞外信號調節激素和機械性痛敏感症的依從性。研究的結果指出，前腦室側視丘中的磷酸化胞外信號調節激素在痛覺模式的小鼠身上劇增，以藥理調控此激素在該腦區的活性可進而舒緩或加劇機械性痛敏感症。最後，在無痛老鼠的前腦室側視丘中，直接以藥理活化胞外信號調節激素可誘發機械性痛敏感症，重複投藥更是造成小鼠發展出慢性機械性疼敏症。這些結果，再再指出 PVA

在痛覺調控的腦內機轉中扮演重要的生理調控功能。我們期待這些發現，除了能對腦部的痛覺生理調控機轉之基礎研究帶來新的思路，更希望有朝能在臨床上為相關疾患之有效療癒帶來新的轉機與曙光。

關鍵詞：前腦室側室丘，機械性痛敏感症，發炎性疼痛，神經病理性疼痛，中樞神經敏感化，胞外信號調節激素。

II. Introduction

The origin of pain and its dual functions fascinate scientists and neurologists in decades. Physiological pain serves as an early-warning protective system attributive to individuals to avoid noxious stimuli and harmful contacts or creating a circumstance disfavor movements and physical contacts to assist injured bodies to repair. On the other hand, the non-protective-related pathological pain results from the abnormality and dysfunction of nervous system and consequently creates the clinical pain syndromes with the greatest unmet need (Woolf, 2010).

20 to 25% of the populations are suffering from the chronic pain in the worldwide investigation (Gold and Gebhart, 2010). Clinical studies on pain management reveal that 10 to 11% of the populations in developed countries and approximately 14% of the US population suffer from widespread chronic muscle pain (DeSantana and Sluka, 2008). Epidemiological studies further illustrate the typical chronic pain patient is mid-aged female (Mogil, 2009). The global market for pain management pharmaceuticals and devices amounted to $19.1 billion in 2008 and is expected to increase to $32.8 billion in 2013 (BCC research, 2009). The increasing demand for analgesics and the importance of pain perception evoke our obligation for a better understanding of nociception pathways.

2.1 The physiological role of pain

Nociceptive pain, a protective, high-threshold pain, detects the high intensity or noxious stimuli, such as heat/cold, sharp, physical force and chemical irritants. When the unavoidable tissue damage happened, the low-threshold pain developed. The individuals exhibit the exaggerative or more intensive pain-related response to the noxious stimuli (hyperalgesia) or innocuous stimuli, such as light touch and warmth (allodynia). This adaptive and protective inflammatory pain usually results from the activation of immune system and causes pain hypersensitivity until the on-going inflammation ceases and the healing occurs. Pathological pain, however, carries on in the conditions after nerve injuries or aberrant function of nerve system even without inflammation in the peripheral tissues (Basbaum et al., 2009; Woolf, 2010).

2.2 Mechanical pain perception

Pain is often but not always associated with mechanical stimuli. The phenomenon of the increase of pain-related response to thermal or mechanical noxious stimulus is referred as thermal and mechanical hyperalgesia respectively. Contrarily, spontaneous pain happens under the situation without any stimulus applied to the subject. Both peripheral and central mechanisms are involved in mediating mechanical pain perception (Lewin and Moshourab, 2004).

2.2.1 The initiation of mechanical nociception

Mechanical nociception is activated and initiated in the peripheral nerve system (PNS). The specialized sensory neurons, nociceptors, detect intense mechanical stimuli and their activation per se is responsible for arising of acute mechanical pain. It has been reported that C-fiber and AM-fiber nociceptors, which have their soma in the dorsal root or trigeminal ganglia, detect intense mechanical stimuli and transduce the nociceptive signals centrally to the dorsal column and trigeminal brainstem sensory subnucleus caudalis (Vc), respectively (Dubin and Patapoutian, 2010). The unmyelinated and free-nerve-ending terminated C-fibers are divided into C-Mechano (C-M), C-Mechanoheat (C-MH), C-Heat (C-H) and C-Mechano insensitive, heat insensitive (C-MiHi) nociceptive fibers, based on their firing patterns and the stimuli to which they respond(Dubin and Patapoutian, 2010). The AM nociceptors are slightly myelinated Aδ fibers which function as nociceptors, whereas the other Aδ fibers (D-hair) are associated with down hair and function as light touch receptors in normal conditions (Lewin and Moshourab, 2004). Molecular approaches also indicate some neuronal peptides and receptors are expressed only in c-fiber, such as substance P (SP), isolectin B4 (IB4), and the Mas-related genes comprised G-protein couple receptors Mrgpr (Tsunozaki and Bautista, 2009). Several classes of ion channels are also identified as involved in primary afferent transduction: voltage gated calcium channels, the Na^+

channels, the transient receptor potential (TRP) channels, the acid-sensing ion channels (ASIC) family, ATP-gated ion channels and the two-pore potassium (KCNK) channels (Dubin and Patapoutian, 2010; Raouf et al., 2010; Tsunozaki and Bautista, 2009).

2.2.2 The transmission of mechanical nociception

Nociceptors convey the nociceptive signals to the central nerve system (CNS) through synaptic connection to the projection neurons and interneurons in the superficial laminae (laminae I, II, and V) of the dorsal horn of the spinal cord (Dubin and Patapoutian, 2010; Tsunozaki and Bautista, 2009). The enhanced neuronal activity in the correspondent projection field of afferent inputs in laminae I and II is observed in inflammatory (Cruz et al., 2005; Ji et al., 1999) and neuropathic pain (Zhuang et al., 2005). A set of projection neurons then transmits nociceptive information to the brain regions responsible for pain perception via brainstem or thalamus before the information reached descending modulation circuits attributable to the motor functions (Basbaum et al., 2009; Schweinhardt and Bushnell, 2010).

2.3 Mechanisms of nociception hypersensitivity

The decrease of the threshold of nociception activation and the augmentation of the intensity of pain-related response are the consequence of the sensitization of the nociception system and the amplification of the input signals. Peripheral sensitization is

defined as the alteration of peripheral neuron properties. It explains the increased peripheral transduction sensitivity and pain hypersensitivity at the stimuli exposure sites (primary hyperalgesia). The maintenance of peripheral sensitization therefore requires on-going peripheral pathology. Central sensitization, changes of the neuron in CNS, on the contrary offers a mechanism accounts for the spontaneous, exaggerated and prolonged pain in response to inflammation pain and pathological conditions, neuropathic and dysfunctional pain. The cardinal feature of the pain underlies central sensitization is that it spreads beyond the inflammatory site (secondary hyperalgesia) (Latremoliere and Woolf, 2009). It has been acknowledged that peripheral sensitization plays a major role in enhanced thermal sensitivity, whilst central sensitization attributes to the major aspects of mechanical nociceptive hypersensitivity (Latremoliere and Woolf, 2009).

2.3.1 Central sensitization

The development of low-threshold hypersensitivity pain and the augmentative intensity of nociceptive response, the up-left shift of response-stimulus (R-S) curve, the increase of spontaneous activities, and the expansion of the projection fields feature central sensitization as the abnormal increased gains of nociception system. The engagement of the functional switch of neurons and circuits in nociceptive pathway in CNS results from the characteristic alterations of these neurons in different aspects: the

membrane excitation facilitation, synaptic capacity and efficacy enhancement, and the inhibitory regulatory attenuation. This happens in both spinal cord and the brain levels. Scientists have endeavored themselves to unveil the mystery of the central mechanism. However, the further they go and the countless deeper and broader questions need to be answered. The consensus of the knowledge of central mechanism is that different pain goes through different modulation pathways and the distinguished stimulator triggers particular nociceptive circuit. Moreover, every single mechanism might recruit some significant molecules and share some, and each circuit would transmit nociceptive information via some common spinal cord layers and brain regions.

2.3.2 Central mechanism in spinal cord

Spinal cord dorsal horn neurons contribute to central sensitization in different pain models have been reported (Basbaum et al., 2009; Ji et al., 2009; Woolf and Salter, 2000). In spinal cord, the interneuron property shift contributes to the alteration of local circuit and the recruit of novel inputs to nociceptive pathways. This mechanism elucidates Aβ fiber-mediated pain. Aβ fiber, the large myelinated low-threshold mechanoreceptor, normally responsive to light touch could participate in nociceptive pathway after sensitization (Latremoliere and Woolf, 2009). The fiber's phenotype switches and the inputs of the fiber were recruited in nociceptive pathway. Consequently, Aβ fiber becomes part of the nociception perception and sending low-threshold inputs from broader reception field and produce pain hypersensitivity in the tissues out of the peripheral insulted site. Additionally, the activation of microglia

and astrocytes in spinal cord also attribute to the central mechanism in neuropathic and inflammatory pain. Been activated by the neuronal peptides and neuron transmitters released by primary afferents, microglia amplifies neighboring neuron activities by forming a glia-neuron amplification loop (Bradesi, 2010).

2.3.3 Central mechanism in brain

Nociceptive information reaches the brain through multiple parallel neuronal pathways. The signals ascend to brain stem and forebrain and directly or indirectly project back to the areas in the brainstem involved in the descending pathway. The spinoparabranchial tract and the spinothalamic tract are two well known ascending pathways (Basbaum et al., 2009; Hunt and Mantyh, 2001; Kuner, 2010) (Ossipov et al., 2010; Schweinhardt and Bushnell, 2010). The nociceptive information ascends to thalamus or central nucleus of amygdala before they are projected to the relevant cortex for further motor functions. Rostral ventromedial medulla (RVM) and the periaqueductal gray (PAG) are involved in descending modulation of pain sensation. PAG receives descending inputs arose from multiple brain regions and communicates to RVM directly or indirectly through locus coeruleus (LC). RVM then projects to the spinal or medullar dorsal horns to facilitate or attenuate the nociceptive transmission and consequently modulate the pain perception.

2.4 The brain circuit for mechanical hyperalgesia

However, with general concurrence, human brain imaging studies have displayed a “pain matrix”, which concluded cortical and subcortical network in normal subjects disregard the different activation patterns across studies. Somatosensory cortices (S1 and S2, respectively), anterior cingulate cortex (ACC), insular cortex (IC), prefrontal cortex (PFC), thalamus, basal ganglia, and cerebellum are included in this matrix (Schweinhardt and Bushnell, 2010). It has been further demonstrated that the brain networks for acute pain in healthy subjects and chronic pain have distinctive features (Apkarian et al., 2005; Lee et al., 2008; Schweinhardt and Bushnell, 2010).

LaMotte (Baumann et al., 1991; LaMotte et al., 1992)first evidenced that the secondary mechanical hyperalgesia induced by capsaicin, an inflammatory pain model, was mediated in the central but not the peripheral system in humans. Hemodynamic and neuroelectrical studies revealed more brain regions other than the ones involved in these pathways in pain perception in healthy or afflicted subjects in response to mechanical stimuli (Apkarian et al., 2005). Lee further reported that during hyperalgesia, there were additional increases of BOLD activity account for the punctuate stimulation found in the subcortical structures matched pain perception are only the brainstem and the thalami in comparison to normal condition (Lee et al., 2008). Additionally, animal studies also reported the changes of fMRI signals in several brain regions including thalamus,

amygdala, and cortex in rat with visceral pain in both genders (Wang et al., 2009; Wang et al., 2008).

Animal studies using different approaches also revealed the importance of central mediation in mechanical hyperalgesia. Evidence shows that most mechanical nociceptive signals are ascended either to parabrachial area in brainstem before sent to the amygdala or the ventral medial nucleus of hypothalamus (VMH). Or, the nociceptive signals are ascended to thalamus and then transmitted to somatosensory cortex through the spino-thalamus pathway (Hunt and Mantyh, 2001; Neugebauer et al., 2004). Previous studies have shown the importance of CeA in the ascending pathway (Carrasquillo and Gereau, 2007; Ikeda et al., 2007; Neugebauer et al., 2004) and the anterior cingulate cortex (ACC), the PAG, and the RVM in the descending pathways (Hunt and Mantyh, 2001; Tillu et al., 2008; Zhang and Zhao, 2010). Carrasquillo and Gereau well illustrated the manipulation of ERK activation in central nucleus of amygdala (CeA) altered the formalin induced mechanical hyperalgesia in mice (Carrasquillo and Gereau, 2007). The pain-related synaptic plasticity alteration account for ERK activity in rats following the induction of nerve injury (Ikeda et al., 2007), arthritis (Han and Neugebauer, 2005; Neugebauer et al., 2003) and in mice with repeated acid-induced chronic muscle pain (Cheng, 2010). Administrating ropivacaine, a local anesthetic, into RVM, Sluka's group also demonstrated that the RVM plays a

role in descending modulation of mechanical hyperalgesia in chronic muscle pain model (Da Silva et al., 2010; Tillu et al., 2008). However, the knowledge of the connection between these nucleuses or between ascending and descending nociceptive pathways and the exact regions which are responsible for mechanical nociception is still unclear. The network of the nociceptive circuit in the brain is therefore still a “black box” waiting to be decoded.

2.5 PVA, a recent revealed locus in central mechanism leads to our hypothesis

In our previous study, a novel region, the anterior paraventricular thalamic nucleus (PVA), which is responsible for acid-induced mechanical hyperalgesia, is identified. PVA, a small region in the midline thalamus, is acknowledged to play a physiological role in modulating circadian rhythm by the means of receives the projections from SCN. On the other hand, increase of nociception correlated neuronal activity markers, c-fos and pERK, have been observed in PVA in several pain models in rodents (Bullitt, 1990; Davies et al., 1997) (Chung et al., 2007; Davis, 2003; Gioia et al., 2001) (Nishii et al., 2008; Zhang et al., 2009).

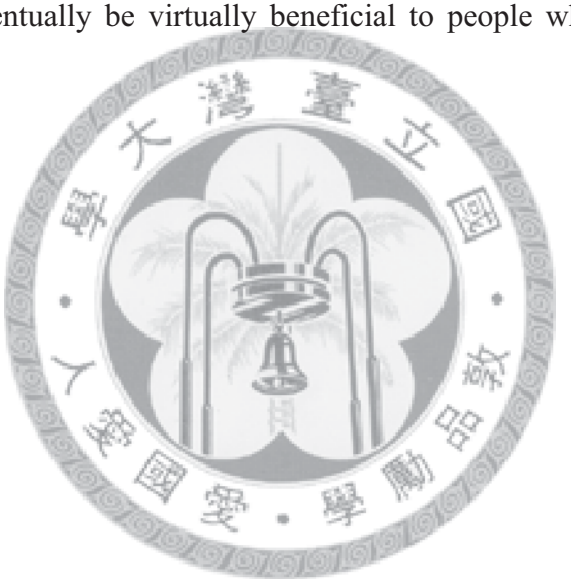
The repeat-acid-induced muscle pain (RAMP) model, invented by Sluka in 2001, is a characterized animal model of long-lasting mechanical hyperalgesia without inflammation and damage in the peripheral tissues (Sluka et al., 2001). The

dysfunctional effects and the enlargement of reception fields it evoked indicate that the central sensitization is accounted for these phenomena. Our previous data shows ERK activation in the PVA is not only acid dependent but also $Ca_v3.2$ T-type calcium channel dependent in this pain model in mouse. Inhibiting ERK activity in PVA stops the mice from developing chronic mechanical hyperalgesia. Intraventricular infusion of $Ca_v3.2$ T-type calcium channel blockers blunts ERK activity in PVA and changes the long-lasting mechanical hyperalgesia induced by the second acid. These finding suggests that PVA is important in acid-induced chronic muscle pain and $Ca_v3.2$ T-type calcium channel mediates the development of long persistent mechanical hyperalgesia via alteration of ERK activity in PVA (Chen et al., 2010). RAMP model induces only mechanical but not thermal hyperalgesia in mice. This property makes us wonder whether PVA also plays an important role in mechanical hyperalgesia in other pain models.

2.6 The aims of this study

To address this question, we aim to determine whether PVA is also involved in the mechanical hyperalgesia in two other pain models, formalin-induced inflammatory pain and SNI-induced neuropathic pain. We first established these two animal models and then determined ERK activity in PVA and its correlation with mechanical hyperalgesia in these two models. We next manipulated ERK activity in PVA using MEK inhibitor,

U0126 and PKC agonist, PDBu, and evaluated the consequent behavioral change and ERK activity by IHC-pERK staining in the naïve and hyperalgesic mice. Accordingly, the importance of ERK activity in PVA in modulating mechanical hyperalgesia in different pain models is illustrated. We expect that the discovery of the importance of PVA in mechanical nociceptive transmission would provide more information in understanding pain perception in brain circuit. Further, with hope, the knowledge we discovered would eventually be virtually beneficial to people who are suffered from pain issues.



III. Materials and Methods

3.1 Chemicals and reagents

Isoflurane, Halocarbon

Hydrochloride, HCl, Merck

U0126, Tocris

U0124, Tocris

dimethyl sulfoxide ,DMSO, Sigma

Phorbol 12,13-dibutyrate, PDBu,Sigma

MES, Sigma

HEPES, J.T. Baker

Xylazine hydrochloride, Sigma

Katamine

Hydrogen peroxide 35%, Merck

Tri-sodium citrate dihydrate, Merck

BSA (albumin bovine), Sigma

Xylene, Sugipath

Alcohol 100%, Sugipath

Vectastain ABC Kit, Vector Lab.

DAB substrate KIT, Vector Lab.

3.2 Solutions

Buffered neutral formalin 10%, Sugipath

Saline: 0.9% NaCl

DMSO 50%: 50%DMSO in saline

Phosphate Buffered Saline, PBS: 137 mM NaCl, 7.7 mM Na_2HPO_4, 2.7mM KCl, 4.5mM KH_2PO_4, pH 7.4

Phosphate Buffered Saline with Tween 20, PBST: 0.05% Tween 20 in PBS.

3.3 Animals

All research performed conformed to National Institutes of Health guidelines in accordance with the guidelines specified by the Institutional Animal Care and Utilization Committee, Academia Sinica. C57/B6 female mice between the ages 8 to 12 weeks were used. All mice were housed in specific pathogen-free conditions in the Institute of Biomedical Sciences, Academia Sinica.

3.4 Animal models

Three animal models were chosen to induce mechanical hyperalgesia in female C57B6 mice. Repeated acid injections induced bilateral chronic mechanical hyperalgesia (Sluka et al., 2001); formalin injection in the hind paw resulted in bilateral inflammatory hyperalgesia in the hind paws (Carrasquillo and Gereau, 2007); spared nerve injury (SNI) induced neuropathic pain (Shields et al., 2003).

3.4.1 Formalin test

Mice were habituated in the recording cages for at least 20 minutes before formalin injection. 10 μl, 5% formalin in PBS or PBS control solution was injected subcutaneously into the plantar surface of the left hind paw. The spontaneous nociceptive behaviors such as flicking, lifting or licking of the injected hind paw were

recorded for one hour after formalin injection. The occurrence of nociceptive behaviors was recorded at the end of every 5 seconds (instantaneous sampling). The total number of nociceptive behaviors in every 5 minutes was accumulated for up to 60 min. The behavior in response to mechanical and thermal stimulus was tested 3 hrs after formalin or PBS injection before their humanitarian sacrificed. The mice went through persistent mechanical hyperalgesia monitoring were humanitarian sacrificed at D7. Most of the mice were with healthy hind limbs and few of them observed with a scare on the epidermis around the injected site 7 days after formalin injection.

3.4.2 SNI model

The mice were anesthetized with vaporized isoflurane (1.5 %) during the surgery. The three distal branches of the sciatic nerve were exposed without damage of the muscle bundles. We used 8-0 nylon sutures to tightly ligate the common peroneal and sural nerves. Approximately a 2 mm-length segment of each ligated nerve was gently dissected distal to the ligature without stretching or touching the tibial nerve. In the sham control mice, the suture went through beneath the targeted nerves respectively without contacting the spared nerve. Layers of muscles and skin were replaced as they were before and the skin was closed neatly by using 5-0 silk suture. Successful surgical operations would not affect the movements of the hind limb. All the operations were done within 15 minutes in different treatmeant groups.

3.4.3 Repeated acid induced muscle pain model (RAMP)

All mice were briefly anesthetized with vaporized isoflurane (1.5 %) before receiving 20 μl acidic (pH 4.0) or neutral (pH 7.2) saline injection in the left gastrocnemius mucle. Acidic saline were injected on Day 0 and Day 3. The control group received neutral saline instead of the acidic saline as the second injection.

35 Behavioral testing

The nociceptive behavior categories include flicking, lifting and licking of the stimulated paw of the testing animal. The behavioral testing is done in blinded manner to pharmacological treatments.

3.5.1 Mechanical hyperalgesia

The measurement of the withdrawal response of experimental mice to von Fray filaments was recorded (North Coast Medical, Morgan Hill, California, USA). Mice were habituated in transparent plastic cubicles on a wire meshed plate form for 20 to 30 minutes. The von Frey filaments were gently applied to the tested mice on each hind-paw until the bending angle was about 30 degrees. Then the nociceptive behaviors were recorded. We applied von Frey filaments at various ranges of bending forces (0.16, 0.4, 0.6, 1, and 1.4 gram) in a progressively increasing manner to

determine the stimulus-nociceptive response curve of the mice. Following this procedure, a von Fray monofilament with 1-g bending force was selected for mechanical hyperalgesia experiments. Ten successful stimuli responses were recorded for each hind paw followed by the percentage of withdrawal responses.

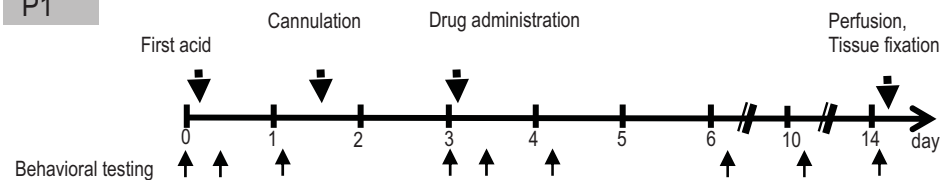
All the experimental and behavioral testing procedures in different models are listed from P1 to P9 as followed. In the repeated-acid model, the behavioral tests were performed on day (D) 0, 1, 3, 4, 6, 10 and 14 in 3-day-interval (3 DI) groups. Behavioral tests were performed twice on D0 (Before and 4hr after first acid injection) and D3 (1hr before second injection (D3a) and 4hr after second acid injection (D3b) when animals received acid injections. The procedures were shown as P1.

In formalin test, the behavioral tests were performed before formalin (or vehicle) injection and 3 hours after injection for each short-term effect experimental groups (P2). For formalin-induced persistent mechanical hyperalgesia experiments, the tests were performed on 3hr, D1, D2, D3 and D7 after formalin injection. In ERK manipulation experiments, the behavioral tests were performed before (a) and at 1 hr (b) and 6 hr (c) after the drug infusion. The mechanical withdrawal tests were performed before (D0a) and at 3hr (D0b), 8hr (D0c) and on D1, 2, 3, 7 after formalin injction for the mice received U0126/U0124 infucsion 2 hr after formalin induction. The test was done at following time points, 3hr, D1, D3a, D3b, D3c, D4, D5, D6 and D7 when the mice

received infusion on D3. The timeline of the experimental procedures are illustrated as P3 and P4 respectively.

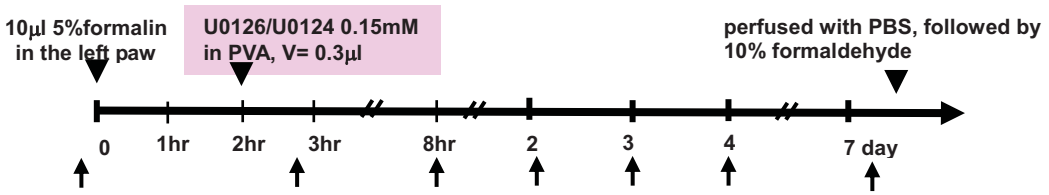
In the SNI model, the behavioral tests were performed before surgery and on D 1, 2, 3, 7 and 14 after SNI or Sham operation. For threshold change testing, we applied von Frey filaments at various ranges of bending forces (0.16, 0.4, 0.6, 1, and 1.4 gram) in a progressively increasing manner at 2 hours after surgery while the mice were sober and fully recovered from the effect of anesthesia. The withdrawal responses to 1-g von Frey monofilament were recorded at 3 hr and 8 hr after nerve dissection, and on D 1, 2, 3, 7, and 14 after the operations in intra-PVA U0126 infusion followed by SNI/sham surgery. For those mice received U0126 infusion 3 days after SNI, the behavioral tests were performed before surgery and on day 1, 3, 4, 5, 6, 7, and 14. The timeline of the experimental procedures are illustrated as P6 and P7.

P1

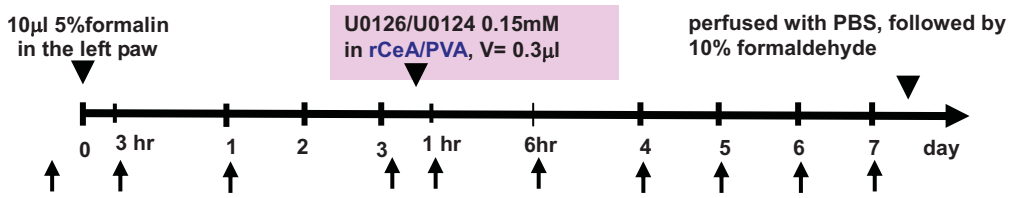


P2

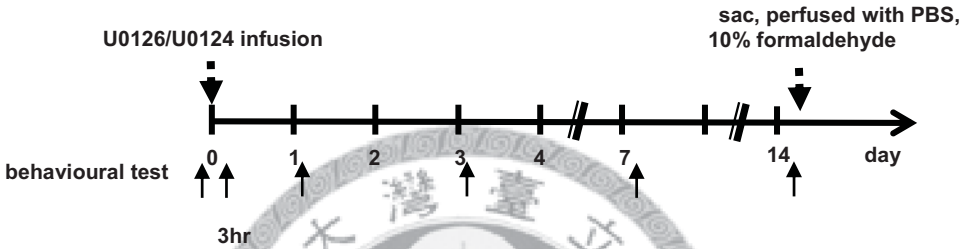
P3



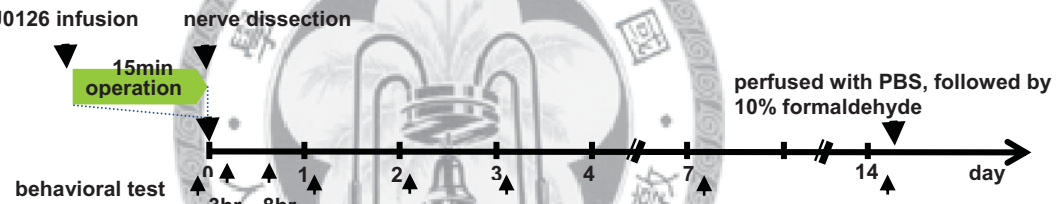
P4



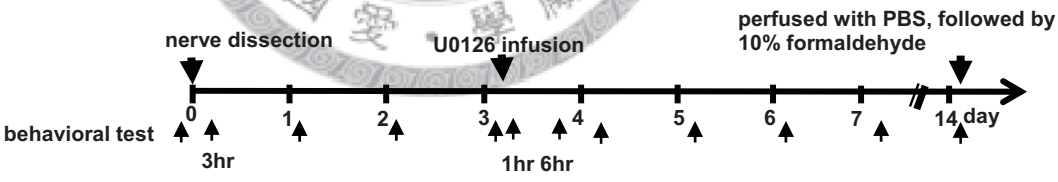
P5



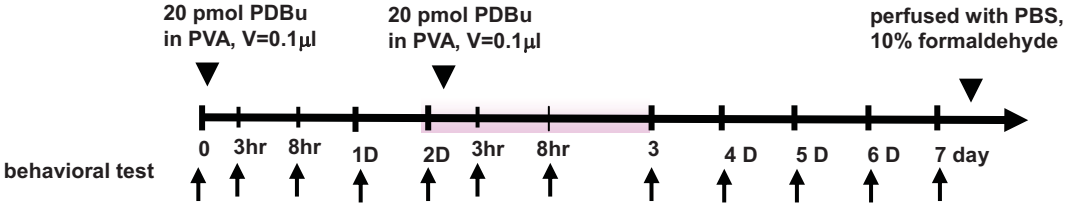
P6



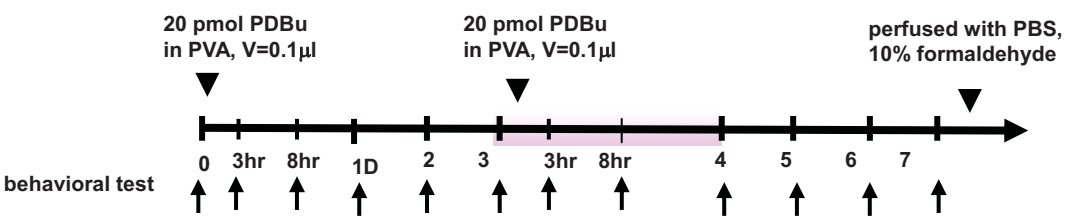
P7



P8



P9



3.5.2 Thermal hyperalgesia

The tested animal was set on to a 50 ℃ thermal plate. The withdrawal latency (s) was measured as the time it takes for the animal to develop nociceptive behavior on the thermal plate.

Three hours after formalin (or PBS) injections, the mice were tested for mechanical hyperalgesia followed by thermal hyperalgesia. The thermal hyperalgesia behavioral tests were also performed at the end of SNI experiments (D14).

36 Cannulation and microinfusion

Mice were anesthetized with katamine/ xylazine and their heads were fixed in a stereotaxic frame and a single particular guide cannula was implanted just above target sites. The cannula was affixed in place by dental cement applied around them onto the skull. A relevant stainless steel flush-fitting stylet was inserted into the cannula. Before infusion, the stylet was removed and an injection cannula with longer length and the same gauge with the stylet's was inserted instead. This cannula was connected to a Hamilton syringe (10 μl) via a section of more than 50 cm length tubing (PE10, Portex Ltd.). The syringe and the tubing were filled with the distilled saline, an air bubble (5 μl), and the infusion solution from the proximal to the distal end subsequently. The air bubble separates the saline and the infusion drug and acts as an index of the success

of the drug infusion as well. (stereotaxic coordinates and cannula sets: 1) intra-PVA, antero-posterior 0.3 mm and dorso-ventral, 3.9 mm from Bregma, 26-gauge 9.5 mm stainless steel cannula, 33-gauge 10 mm infusion cannula; 2) above the right central nucleus of amygdale (RCeA), antero-posterior 1.7 mm, left-right -2.6 mm and dorso-ventral, 3.7 mm from Bregma, 23-gauge 8 mm stainless steel cannula, 30-gauge 8.5 mm infusion cannula)

A reported MEK inhibitor, U0126 (Sigma, St. Louis, Missouri, USA), and a PKC agonist, PDBu (Bristol, UK), were infused into PVA to inhibit and activate ERK expression, respectively. 10 mM U0126 and 3 mM PDBu were dissolved in 100% DMSO as stock solutions and diluted 1:1 in saline (0.9% sodium chloride) before applied to the mice. 50% DMSO/saline (1:1) were used as a vehicle control.

3.6.1 Acute infusion

At the time points as previous described in formalin test and SNI models, the drug was infused into PVA or RCeA of the anaesthetized mice acutely. A previously modified 33-guage injection cannula and a syringe pump (KD Scientific) were used in this acute infusion. After infusion, the injection cannula was kept in place for an additional 2 min to allow the drug to diffuse before cannula removing. (The mice were surrounded by warm air for having an expeditious recovery)

3.6.2 Microinfusion in repeated acid induced muscle pain model

On D 3 or D 5, 0.3 μl of the drug were infused over a 3-min stint using a syringe pump (KD Scientific) and the injection cannula were kept in place for an additional 2 min to allow the drug to diffuse. Followed U0126 but not PDBu intra-PVA infusion, the second acid was then applied immediately. At the end of each experiment, the RCeA or intra-PVA infused brains were harvested to examine the infusion sites and ERK activity.

3.7 Immunohistochemistry.

The deeply anesthetized mice were perfused transcardially with PBS, followed by 10% neutral buffered formaldehyde solution at 10 minutes or 2 hours after the second intramuscular injection and the end point of each set of experiments. The brain was dissected, fixed and embedded in paraffin blocks. Five-μm-thickness sections were cut coronally and then mounted on the slide. The slide was immersing in boiling 0.01 M citric acid for 40 min to perform tissue antigen retrieval. Sections were then treated with 3% H_2O_2 for ten minute and block with 2% normal goat serum and 2% BSA in PBST for one hour at room temperature. After that, sections were incubated with pERK antibody (1:100, in serum blocking solution, Cell signaling Technology, Denvers,, Massachusetts, USA) at 4 °C overnight. After their being washed with PBS, biotinylated anti-rabbit IgG was used as a secondary antibody, and the ABC method was used for

signal detection (Vecstain ABC KIT, Vector Laboratories, Inc., Burlingame, California, USA).

3.8 Statistical analysis

Mechanical withdrawal ratios of the testing paws were tested for differences between control and experimental groups with a non-parametric Kruskal – Wallis test. The dependence of withdrawal ratio upon time of each group was assessed with a signed rank test. The withdrawal ratios for differences across time and between groups were tested with one way repeated measures ANOVA, followed by a post hoc testing using Holm-Sidak method between groups and paired t-test across time. The withdrawal ratio in formalin test and RAMP models were presented as the mean ± SEM of the average of both hind limbs and in SNI model was presented as the mean ± SEM of each limb. Data were considered significant if $p < 0.05$.

IV. Results

4.1 PVA is involved in formalin induced inflammation pain

To determine whether PVA is involved in the formalin-induced persistent mechanical hyperalgesia caused by the peripheral inflammation, we first established formalin test in female C57B6 mice. As shown in figure 1A, formalin induced biphasic spontaneous responses with the first phase appearing within the first 5 min and the second phase arising during 20 to 40 min. 3 hr after injection, the mice received formalin injection exhibited significant increase of mechanical withdrawal ratio compared to the basal response while the PBS control group showed no change in the withdrawal ratio (Figure 1B). These results are consistent with the reported ones indicating the success of formalin-induced inflammatory pain (Carrasquillo and Gereau, 2007; Kolber et al., 2010; Saddi and Abbott, 2000).

Using phosphorylated extracellular signal-regulated kinase (pERK) as a nociception-related neuronal activity marker, we investigated the neuronal activity in PVA. Gereau's group has demonstrated increased pERK staining in CeA at 3 hrs after formalin injection and alteration of ERK activity in CeA modulates formalin-induced mechanical hyperalgesia in rodents (Carrasquillo and Gereau, 2007; Kolber et al., 2010). Accordingly, we examined the level of pERK staining in both PVA and CeA regions at 3

hrs after formalin or PBS injection. As shown in Figure 1C, enhanced pERK signals were observed in both PVA and CeA in the formalin injected group compared to the PBS control group. Formalin-induced mechanical hyperalgesia is known to last for 3 weeks (Li et al., 2010). To determine the ERK activity in these brain regions during the persistent hyperalgesic phase, we performed IHC-pERK staining 3 days after formalin injection. Interestingly, pERK positive signals were still detectable in PVA but not in CeA 3 days after formalin injections (Figure 1C, lower panels). These data suggested that neuronal activity in PVA increased in formalin-induced inflammatory pain and this intensified neuronal activity lasted at least for 3 days.

4.2 Manipulation of ERK activity in PVA alters mechanical but not thermal hyperalgesia

To investigate whether ERK activity in PVA is important for the generation of the mechanical hyperalgesia induced by formalin, we blocked ERK activity via intra-PVA infusion of U0126, a reported MEK inhibitor, at 2 hr after formalin injection as procedure P2. U0126 but not its' inactive analogue U0124 decreased the mechanical hyperalgesia at 3 hr after formalin injection (Figure 2A). These results indicate that increased ERK activity in PVA is required for the formalin-induced mechanical hyperalgesia.

It has been reported that formalin injection induced considerable thermal allodynia and hyperalgesia evoked by the on-going peripheral inflammation. Accordingly, we examined the thermal response after intra-PVA infusion of drugs after von Frey filament test. As shown in Figure 2B, formalin induced thermal hyperalgesia as illustrated by the shortening of withdrawal latency on the thermal plate. Neither U0126 nor U0124 infusion changes the thermal hyperalgesia induced by formalin.

Taken together, inhibition of ERK activity in PVA altered mechanical but not thermal hyperalgesia in formalin test. This further indicates that neuronal activity in PVA is important for the mechanical hyperalgesia induced by formalin.

4.3 The inhibition effect of U0126 in long-term persistent mechanical hyperalgesia

Formalin test is known to induce persistent mechanical hyperalgesia lasting for 2 weeks in mice and 3 weeks in rats (Fu et al., 2001; Li et al., 2010). We then asked whether inhibition of ERK by U0126 in PVA has any effect on the persistent mechanical hyperalgesia in formal test. We first confirmed that the formalin-induced long-term mechanical hyperalgesia lasted at least for 1 week in female C57B6 mice (Figure 3A). We then infused U0126 into PVA 2 hr after formalin injection and then performed behavioral test for mechanical hypersensitivity at 3hr, 8hr, 1d, 2d, 3d and 7d after formalin injection (procedure illustrated as P3). As shown in Figure 3B,

formalin-induced mechanical hyperalgesia was transiently reduced one hour and 6 hours after U0126 infusion (see insert of Figure 3B). Nevertheless, both U0126 and U0124 had no long-term effect on the mechanical hyperalgesia induced by formalin (Figure 3B). These results suggest that inhibition of ERK activity in PVA 2hr after formalin injection could not prevent the development of long-term hyperalgesia induced by formalin.

We then asked whether inhibition of ERK activity in PVA could reverse the existing long-term mechanical hyperalgesia induced by formalin. Since pERK positive signals in PVA can be detected 3 days after formalin injection, we examined the effect of U0126 infusion in PVA on the mechanical hyperalgesia 3 days after formalin injection (procedure illustrated as P4). Similar to those shown in Figure 3B, U0126 but not U0124 infusion at D3 resulted in an immediate transient inhibition effect (significantly different between 1hr and 6hr after U0126 infusionl, † $p<0.001$, Figure 3C). Interestingly, the effect of U0126 in attenuating the mechanical hyperalgesia remained until the end of the experiment at D7 (* $P<0.001$ significantly suppressed compared to the U0124 control goup). On the other hand, U0124 infusion at D3 has no effects on the existing persistent mechanical hyperalgesia induced by formalin. These results suggest that inhibition of ERK activity in PVA abolishes the withdrawal ratio transiently and followed by attenuating the existing mechanical hyperalgesia

induced by formalin.

Besides, when we looked into the thermal response of all the treatment groups at the final day of experimental procedure, all the U0126/U0124 treated groups displayed considerable reduced thermal withdrawal latency, which is consistent with the formalin induced latency reduction(Figure 3D). This result further confirmed the null effect of ERK activity in PVA on formalin-induced thermal sensitization.

It has been demonstrated that ERK activity in CeA is involved in the early stage of formalin-induced mechanical hyperalgesia (Carrasquillo and Gereau, 2007), and our previous study suggested that the nociceptive information reached PVA probably through CeA (Chen et al., 2010). We asked whether infusion of U0126 into RCeA 3 days after formalin injection has any effect on the later stage of persistent mechanical hyperalgesia. As shown in Figure 4, neither U0126 nor U0124 infusion into RCeA has any effects on the existing persistent mechanical hyperalgesia induced by formalin. The infusion in RCeA on D3 presented mute effect on mechanical hyperalgesia further emphasized the importance of ERK activity in PVA is not only for the initiation but also the maintenance phase of the formalin-induced persistent mechanical hyperalgesia.

4.4 The effect of U0126/U0124 infusion in naïve mice

To clarify the inhibition effect of U0126 on mechanical hyperalgesia is from the

pharmacological effect of U0126 per se but not the influence of operational procedures, we infusion either U0126 or U0124 into PVA of naïve mice. As shown in Figure 5, neither U0126 nor U0124 infused into PVA has any effects on the mechanical hyperalgesia up to 14 days in naïve mice. The results clearly illustrated that the mechanical sensitivity of the animal was not affected by intra-PVA infusion procedure.

4.5 PVA is involved in SNI-induced neuropathic pain

To examine whether PVA is also involved in SNI-induced neuropathic pain, we established SNI model in female C57B6 mice followed the method modified by Basbaum's group (Shields et al., 2003). SNI surgery induced unilateral persistent mechanical lasted at least for 14 days (Figure 6A). The withdrawal response of the contralateral, non-operated, limb was unaffected. Interestingly, sham operation also resulted in a 2-day stint of mechanical hyperalgesia peaked at D1 and declined back to basal level at D3 on the ipslateral limb (Figure 6B). The increase of mechanical withdrawal ratio of the sham-operated limb indicates that the post-operative effect also induced temporal mechanical hyperalgesia. This further suggests that the early stage of mechanical hyperalgesia induced by SNI surgery was under the commands of both surgery-induced inflammatory pain and neuropathic pain.

We further examined the neuronal activity in PVA using pERK as a marker at 3 hr,

2 days and 3 days after surgery in both SNI and sham operated groups. IHC-pERK staining demonstrated the strong pERK positive signals in PVA and CeA in both operated groups albeit the signals were relatively condensed in CeA in SNI-operated group 3 hr after surgery (Figure 6C, upper panels). 2 days after surgery, the pERK positive signals were still present in the PVA and CeA of SNI group. Contrarily, in the sham operated group, the pERK signals can only be detected in the PVA but not the CeA 2 days after surgery (Figure 6C, mid-panels). On the day 3, attenuated pERK signals were observed in PVA but not CeA of SNI group while there was no detectable pERK signal in the sham-operated groups (Figure 6C, lower panels). These data suggests that neuronal activity in PVA increased in SNI-induced neuropathic pain and this enhanced neuronal activity lasted for at least 3 days.

4.6 The inhibition effect of U0126 intra-PVA infusion in SNI model

To investigate whether inhibition of ERK activation in PVA could prevent the development of SNI-induced mechanical hyperalgesia, we first determine the onset timing of mechanical hyperalgesia induced by SNI but not the shame operation. Because it takes about 2 hours for the mice to completely recover from the surgery and regain the strength to lift up their operated limbs, the first time point we performed the behavioral test is 2 hour after the surgeries. As shown in Figure 7, SNI but not the sham-operated group already induced mechanical hyperalgesia 2 hour after the surgery.

Since the half life of U0126 is around 2 hour *in vivo*, we decided to infuse U0126 before the surgery (procedure P6). As shown in Figure 8A, intra-PVA U0126 infusion reduced the mechanical hyperalgesia at 3hr and 8 hr but not those measured at later time points after SNI surgery. Interestingly, U0126 infusion obviously blunted the mechanical hyperalgesia under the influence of post-operative effect in the sham-operated group (Figure 8B). These results suggest that inhibition of ERK activity in PVA can only attenuate the mechanical hyperalgesia transiently but not prevent the development of long persistent hyperalgesia induced by SNI surgery.

To investigate whether inactivation of ERK in PVA could attenuate the existing SNI-induced mechanical hyperalgesia, we infused U0126 into PVA 3 days after surgery (procedure P7). U0126 but not U0124 infusion abolished the unilateral mechanical hyperalgesia induced by SNI surgery dramatically (Figure 8C, D). The suppressed mechanical hyperalgesia caused by U0126 infusion on day 3 lasted up to 14 days after SNI surgery (Figure 8E). These results suggest that inhibition of ERK activity in PVA can abolish the existing persistent mechanical hyperalgesia induced by SNI

4.7 ERK activation in PVA elevated chronic mechanical hyperalgesia in RAMP model

In our previous study, we demonstrated that ERK activation is important in RAMP model and inhibition of ERK activity in PVA changed the mechanical hyperalgesia

induced by repeated-acid injection. We ask whether ERK activation in PVA alone without the second muscle acid injection would change mechanical hyperalgesia (procedure P1). As shown in Figure 9B, three days after the 1st acid injection in the muscle, intra-PVA PDBu but not vehicle infusion induced the chronic mechanical hyperalgesia in the absence of the 2nd acid injection. These data suggest that activation of ERK in PVA facilitates the mechanical hyperalgesia. They further support the idea that ERK activation in PVA plays an important role in modulating mechanical hyperalgesia.

4.8 ERK activation in PVA in naïve mice induced mechanical hyperalgesia

Concluded from all the presented results and our previous study, ERK activation in PVA plays an important role in mechanical hyperalgesia in formalin-induced inflammatory pain, SNI-induced neuropathic pain and acid-induced chronic muscle pain. The next question we asked is whether activation of ERK in PVA is enough to induce mechanical hyperalgesia in the absence of peripheral insults. To answer this question, we infused PDBu into PVA without any peripheral insult. As shown in Figure10A, intra-PVA infusion of PDBu increased pERK staining in PVA but not in CeA 3hr after infusion. PDBu infusion resulted in a short period of mechanical hyperalgesia which lasted for around one day (Figure 10B) without affecting the thermal sensitivity of the mice (Figure 10D). Contrarily, infusion of vehicle control had no effect on both

mechanical and thermal response (Figure 10B). The increase of mechanical withdrawal ratio increased significantly 3 hr after infusion compared to its basal (Figure 10C). These results indicate that ERK activation in PVA alone is enough to induce mechanical but not thermal hyperalgesia in the absence of peripheral injury. These experiments also pinpoint the crucial role of PVA in mechanical hyperaglesia in brain circuit.

In the presence of peripheral insults, manipulation of ERK activity in PVA changed the persistent mechanical hyperalgesia in different models (Figure 3, 8 and 9). However, infusion of PDBu in PVA once elicited a stint of mechanical hyperalgesia that lasted only around one day (Figure10B). We then ask whether it is possible to induce long-lasting mechanical hyperalgesia by repeated infusion of PDBu in PVA. We infused PDBu into PVA twice separated from the 1st infusion by 2 days or 3days, procedures illustrated as P8 and P9. As shown in Figure 11, repeated PDBu infusion with 3-day interval (3DI) (filled circle) induced two 2-day stints of mechanical hyperalgesia as the consqence of infusion. On the other hand, repeated PDBu infusion with 2-day interval (2DI) (open circle) induced a sustained mechanical hyperalgesia lasted up to 14 days (Figure 11). These results clearly demonstrated that ERK activation in PVA pharmacologically is able to induce a persistent mechanical hyperalgesia in the absence of peripheral injury and ERK activation in PVA facilitates to mechanical hyperalgesia.

V. Discussion

5.1 The role of PVA in mechanical hyperalgesia

Our data provides the evidence that PVA plays an important role in the central mechanism of mechanical hyperalgesia. Activation of ERK in PVA induced mechanical hyperalgesia in naïve mice indicates that PVA is an important pain matrix for mechanical nociception modulation in central mechanism (Figure 10). ERK activation in PVA is not only involved in the initiation of mechanical hyperalgesia, but also participated in the development of chronic mechanical hyperalgesia. Repeated PDBu infusion in PVA with 2-day interval generated prolonged mechanical hyperalgesia for at least 5 days in mice (Figure 11B) gave the absolutely support to this conclusion. Interestingly, the increased mechanical withdrawal ratio seemed to remain at the level before the second PDBu infusion on D2. It would be interesting to know whether we could mimic the formalin-induced persistent mechanic hyperalgesia by microinfusing PDBu continuously or infusing the second PDBu on D1 when the mice still display the higher intensity of the withdrawal response.

We demonstrated that inhibition of ERK activity in PVA on D3 not only attenuated formalin-induced prolonged mechanical hyperalgesia (Figure 3E), but also blunted SNI-induced persistent mechanical hyperalgesia (Figure 8D). Inhibition of PVA

neuronal activity by U0126 in the later phase changed the chronic nociceptive behavior induced by different pain models evidenced that PVA plays a role in the maintenance of persistent mechanical hyperalgesia.

Although our previous study demonstrated that U0126 infusion before the second acid diminished the develop of mechanical hyperalgesia, administrating U0126 into PVA before SNI surgery and 2 hr after formalin injection produced only a transient inhibition effect on withdrawal ratio response to 1-g von Frey filament. This could be explained by short physiological half life of U0126 and the on-going peripheral inflammation in early stage of SNI and formalin model but not in RAMP.

In RAMP model, replacement of the 2nd acid injection with intra-PVA infusion of PDBu on day 3 after first acid injection also induced the long persistent mechanical hyperalgesia (Figure 9). The increase of mechanical nociceptive intensity of repeated PDBu infusion with 2-D interval (opened circle, Figure 11) is relatively tenuous compared to this augment of intensity. The difference between these two experiments is the priming effect from the first acid injection in the RAMP model. The first acid might change the properties of nociception responsible neurons in PVA and these neurons are subsequently primed. When the second stimulus reached PVA, the neurons in “primed state”(Hucho and Levine, 2007) initiated the chronic mechanical hyperalgesia vigorously. Our previous study revealed the role of $Ca_v3.2$ T-type calcium channel in the

initiation of the chronic mechanical hyperalgesia. It regulates ERK activation and further facilitate to the development of chronic hyperalgesia in RAMP model. In current study, we showed that as long as the neurons are primed, ERK activation in PVA is enough to trigger chronic mechanical hyperalgesia in the absence of further peripheral insult. Direct PDBu infusion instead of the second acid in $Ca_v3.2$ T-type calcium channel deficient mice will further support this idea.

On the contrary, the peripheral insult is necessary for neurons to be primed. Repeated intra-PVA PDBu infusions separated by 3 days without peripheral insults induced two transient increases of withdrawal incidence following the microinfusions (Figure 11). However, similar second PDBu infusion with 3 days apart from the first acid injection induced persistent mechanical hyperalgesia (Figure 9). The second stimuli are the same, however, the consequence they caused were different. This also suggests the peripheral insult transmits priming signals to prime the neurons in PVA or the peripheral insult triggered the priming system in CNS. Joseph and Levine (Joseph and Levine, 2010) reported that mechanical hyperalgesic priming occurs restrictively in IB4-positive nociceptors in inflammatory pain in rats further supports the importance of the information from peripheral for priming effect. Besides, the role of peripheral insult in priming effect is also consensus to the understanding of the physiological role of pain. When the adaptive and protective role of pain enforces, the first peripheral insult is

necessary to trigger the exaggerate response to stimulus for avoiding the contact and protecting the wounded body.

In previous study and current study, our data proved that PVA plays a role in modulating mechanical but not thermal hyperalgesia in inflammatory pain (formalin), and pathological pain, includes neuropathic pain (SNI) and dysfunction pain (RAMP). However, we have no clue about whether PVA also modulated spontaneous pain induced by these treatments. To patients with chronic pain conditions, irritating spontaneous pain composes their most complaints. The animal models for studying spontaneous pain began in the recently years. The researchers use ultrasound vocalization (Kurejova et al., 2010) and conditioned place preference to concomitantly determine the presence of tonic pain (King et al., 2009) in rodents. Albeit the central mechanism of spontaneous pain probably differ from evoked pain, it would be interesting to know the role of PVA in it and it could have better chance to attribute to people suffered from it.

The well appreciated physiological role of PVA is modulating of circadian rhythm. It has been reviewed that circadian rhythm has an effect on pain perception and efficacy of analgesics. Different types of pain also show different circadian pain rhythms in patients (Junker and Wirz, 2010). Thermal nociceptive sensitivity changed in male mice following the circadian rhythm is reported by Konecka and Sroczynska (Konecka and

Sroczynska, 1998). Our data demonstrated that PVA plays a role in modulating of mechanical hyperalgesia. We wonder whether PVA also plays as the connection between circadian rhythm and pain sensation and attribute to the development of circadian pain rhythms. This will need further experiments to be answered.

5.2 pERK, the nociceptive marker in central sensitization

To evaluate neuronal activity in response to nociception signaling in PVA, we used pERK as a neuronal nociceptive activity marker. Our results manifest that ERK activation in PVA involves in central mechanism of mechanical hyperalgesia in formalin-induced inflammatory pain and SNI-induced neuropathic pain. ERK is specifically activated by noxious stimuli in CNS, essential for central sensitization, increased sensitivity, and responsible for development and maintenance of persistent pain in different pain models (Carrasquillo and Gereau, 2007; Chen et al., 2010; Cruz et al., 2005; Gao and Ji, 2010; Ji et al., 1999). The robust, dynamic activation of ERK and its role in central sensitization makes pERK a better nociceptive marker than c-fos (Gao and Ji, 2010).

ERK activation is involved in the maintenance of persistent pain in inflammatory, neuropathic pathological pain (Gao and Ji, 2010; Kuner, 2010). In spinal cord, ERK activation is involved in central sensitization via increasing NMDA and AMPA

receptors activities and reducing the activity of Kav4.2 potassium channels at the posttranslational level. At the transcriptional level, ERK up-regulates its target genes expression, such as: c-fos, NK-1, Prodyn, Cox-2 and TrkB, via its downstream transcription factor, cAMP-response element binding protein (CREB). The subsequent effects are spinal cord wind-up, long-term potentiation, and long-term neuronal plasticity.

In brain, how ERK activation is involved in the central sensitization is unclear. The consensus is that ERK activation regulates the phosphorylation of CREB. However, CREB can be regulated through multiple kinetic cascades, such as PKC-, PKA-, and CMKII-dependent pathways. ERK activation is therefore not the only explanation for increasing of CREB activity.

5.3 Nociceptive Pathways

5.3.1 The signals in CeA

Gereau's group (Carrasquillo and Gereau, 2007; Kolber et al., 2010) has well demonstrated the ERK is activated in CeA 3 hr after formalin intra-plantar injection and CeA plays a role in formalin induced mechanical but not thermal hyperalgesia. In our hand, ERK activation in CeA was confirmed by IHC-pERK staining. Interestingly, pERK signals disappeared 3 days after formalin and SNI induction while the mice

displayed persistent mechanical hyperalgesia in both models (Figure 1C, 6C). This suggests that indeed CeA is important for mechanical hyperalgesia in formalin induced persistent pain rather in the early stage than in the long term stage. Inhibition ERK activity in RCeA 3 days after formalin induction presented no effect on formalin-induced hyperalgesia (Figure 4) further emphasizes that the importance of CeA in nociception modulation is in the early stage and PVA plays more crucial role in the later stage of formalin-induced mechanical hyperalgesia.

It has been reported that CeA has a dual roles in nociception sensations. One is the the ascending signals reaches CeA before been transmitted to forebrain. This will activate neurons in CeA and facilitate the nociception sensation (Kuner, 2010; Neugebauer et al., 2004). On the other hand, CeA is also involved in the descending modulation (Neugebauer et al., 2004; Ossipov et al., 2010). The descending signals from cortex projects to CeA through lateral amygdala (LA) and basal lateral of amygdala (BLA). CeA further transmits descending modulation signals to PAG for inhibition nociception sensation. Different pains induced different local mechanism in amygdala. It is unclear what caused the signals in CeA disappeared 3 days after induction while the on-going peripheral condition kept sending inputs to CNS in formalin and SNI models. One wild guess is that the dual signals, ascending facilitation and descending inhibition, play balance in CeA then.

5.3.2 The brain circuits

It has been reported that nociception information ascends via spino-parabrachial tract to the CeA, and CeA then sends outputs to thalamus in inflammatory pain and many neuropathic pain models ascend their information through spinothalamic tract into lateral thalamus directly (Kuner, 2010). The signals ascend to lateral thalamus and then to cortex topographically and provide somato-sensory discrimination and pain intensity information. The other signals go through limbic system is attributable to affective component of pain. Nevertheless different pain go through different pathways, they all probably send signals to cortex through thalamus.

In RAMP model, our previous study suggested that nociceptive information probably reach PVA from CeA through spino-parabrachial pathway (Chen et al., 2010). Additionally, Cheng, et al. demonstrated that the synaptic plasticity changed in the neurons in CeA after the second acid injection in mice (Cheng, 2010). U0126 infusion in RCeA before the second acid alters the chronic mechanical hyperalgesia into a transient increase of withdrawal ratio. IHC-pERK staining demonstrated that inhibition ERK in RCeA blunts the ERK activation in PVA and LCeA as well. These results further support the idea that the outputs from CeA were eventually send to PVA.

Our study is the groundwork for studying the role of PVA in the “pain matrix”.

Retrograde labeling has shown that PVA doesn't receive signals only from CeA (Novak et al., 2000; Otake et al., 1995)) it also receives signals from PAG (Krout and Loewy, 2000). PAG is known as a descending modulation nucleus and the activation of PAG neurons contributes of descending inhibitory pathway. Nevertheless, PAG-thalamus circuits also play multiple roles in physiological modulation, which includes autonomic, nociceptive, forebrain circuits associated with defense and emotional responses (Krout and Loewy, 2000; Ossipov et al., 2010). Besides, PVA projects the outputs to the extended amygdale, BL and the medial prefrontal cortex was reported by Li and Kirouac (Li and Kirouac, 2008). These forebrain regions are known involved in forebrain circuits attribute to emotional and aversion component of nociception and other afferent responses (Neugebauer et al., 2004; Ossipov et al., 2010). It is unclear whether PVA also involves in nociception descending modulation and how and where exactly PVA transmits the nociceptive information.

Our finding clearly identified the critical role of PVA in the mechanical hyperalgesia. PVA contributes to mechanical modulation through increase or suppress ERK activity. ERK activation in PVA facilitates to the development and maintenance of mechanical hyperalgesia and inhibition of ERK activity in PVA attenuates the increase of the intensity of withdrawal response. These data suggests PVA involves in the "pain matrix", and ERK activity in PVA reveals the potential central mechanism in pain

perception in brain. To be our ambitions, these data would offer a potential target and mechanism in clinical approach to alleviate evoked pain.

VI References

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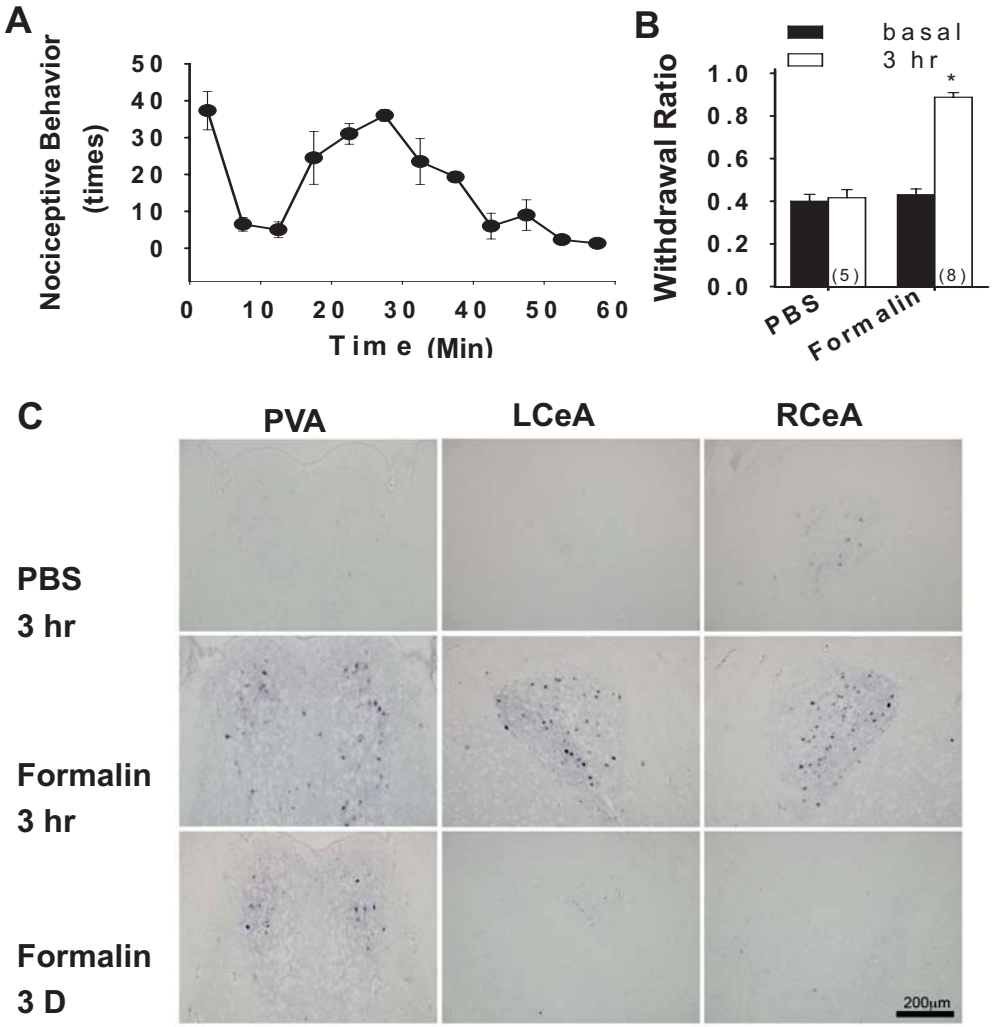

Figure 1 Nociceptive behaviors and ERK activation in PVA and CeA in formalin test. **A**. Formalin injection induced typical bi-phased spontaneous pain in C57B6 mice. **B.** Mechanical hyperalgesia increased significantly 3 hr after 5% formalin injection compare to its basal level. **C.** Substantial IHC-pERK positive signals were detected in both PVA and amygdala regions 3 hr after formalin injection. 3 days after injection, pERK positive signals vanished whereas the pERK signals in PVA sustained. (*$p<0.05$, paired T-test, sample size is shown in parenthesis.)

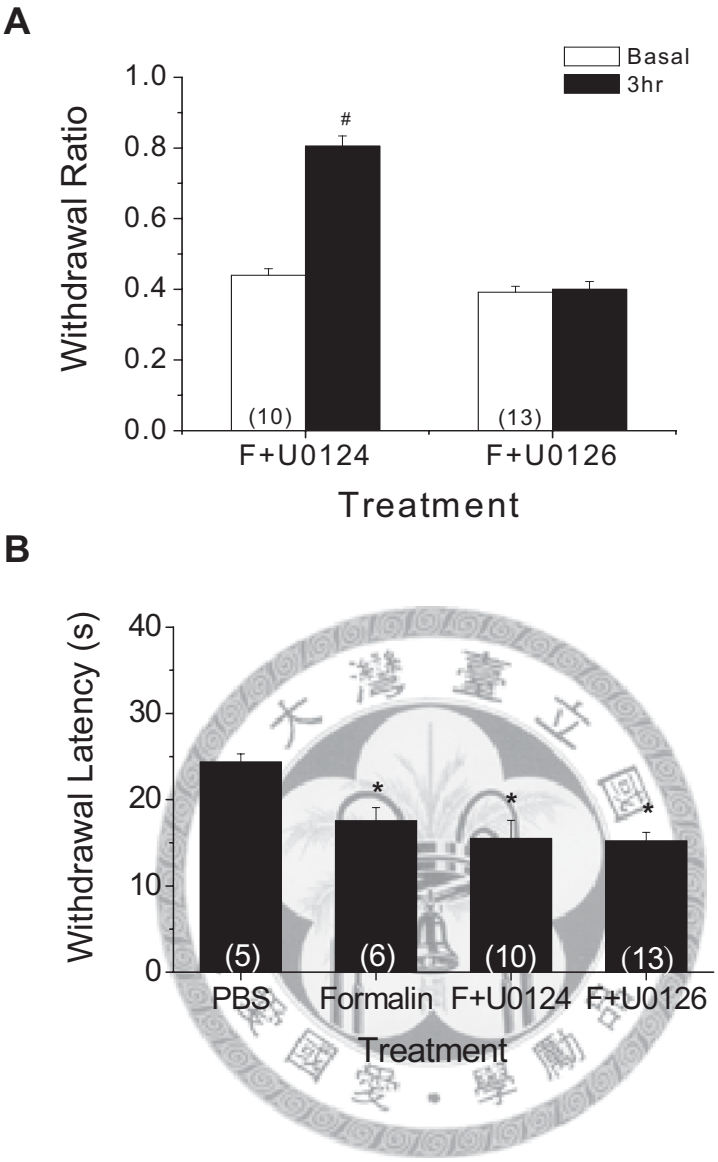

Figure 2 Manipulation of ERK activity in PVA changes mechanical but not thermal hyperalgesia. A. Intra-PVA infusion of PDBu exacerbates mechanical hyperalgesia and infusion of U0126 alleviates mechanical hyperalgesia 3hr after formalin induction (# $p<0.05$, paired T-test, compared to basal level). **B.** Thermal withdrawal latency decreases dramatically after formalin injection. Neither U0126 nor U0124 infusion changes the thermal withdrawal latency (* $p<0.05$, paired T-test, compared to PBS control.).

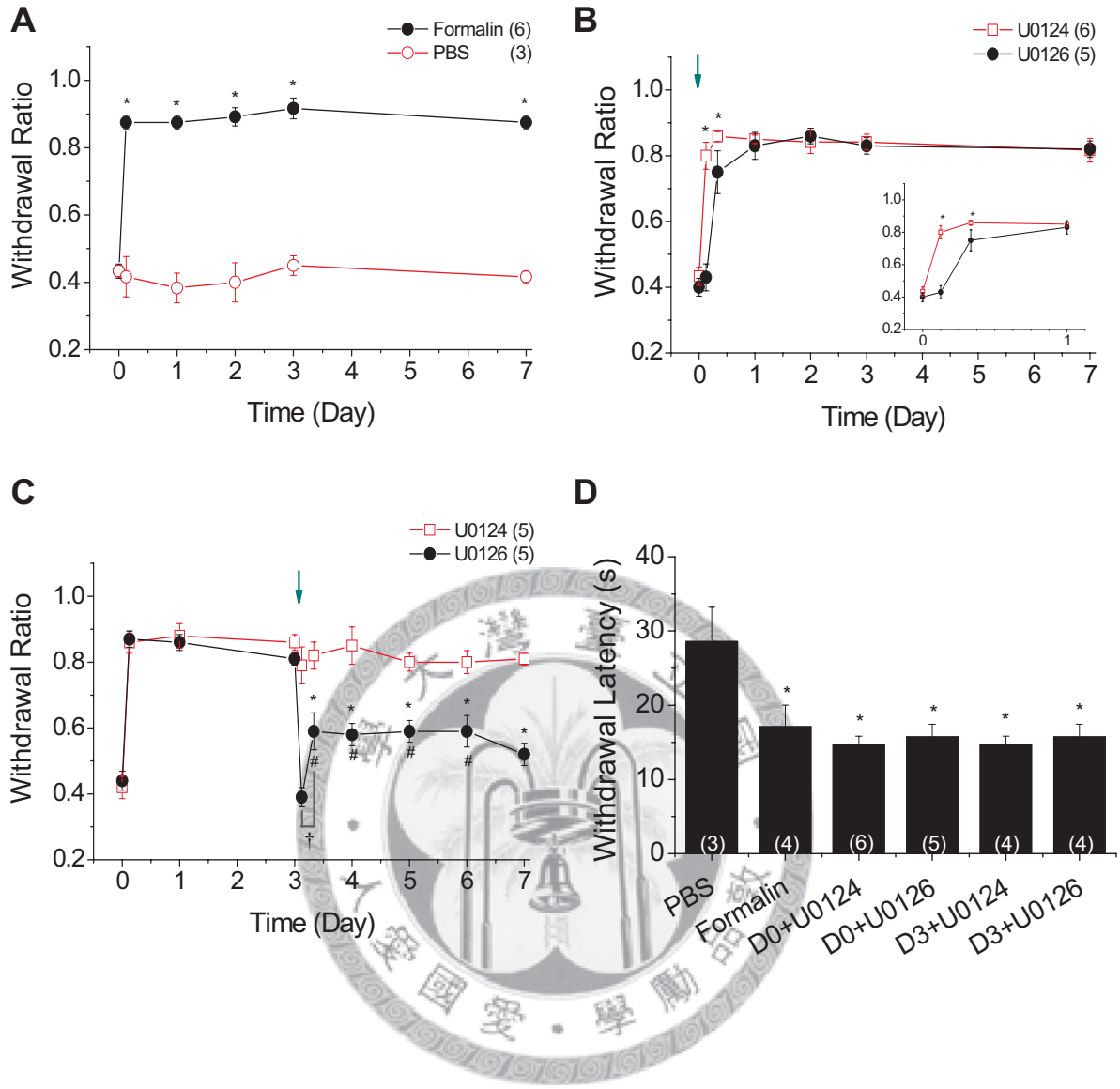

Figure 3 The effect of intra-PVA infusion of U0126 on persistent mechanical hyperalgesia. A. Formalin induced long-lasting mechanical hyperalgesia. **B, C.** U0126 infusion (blue arrow) at 2hr **(B)** or 3 days **(C)** after formalin injection reduced mechanical hyperalgesia transiently. **D.** Formalin induced thermal hyperalgesia is not affected by U0126 or U0124 intra-PVA infusion (* significantly different from the control group; # significantly greater than basal; † significantly different between groups across time.) **.**

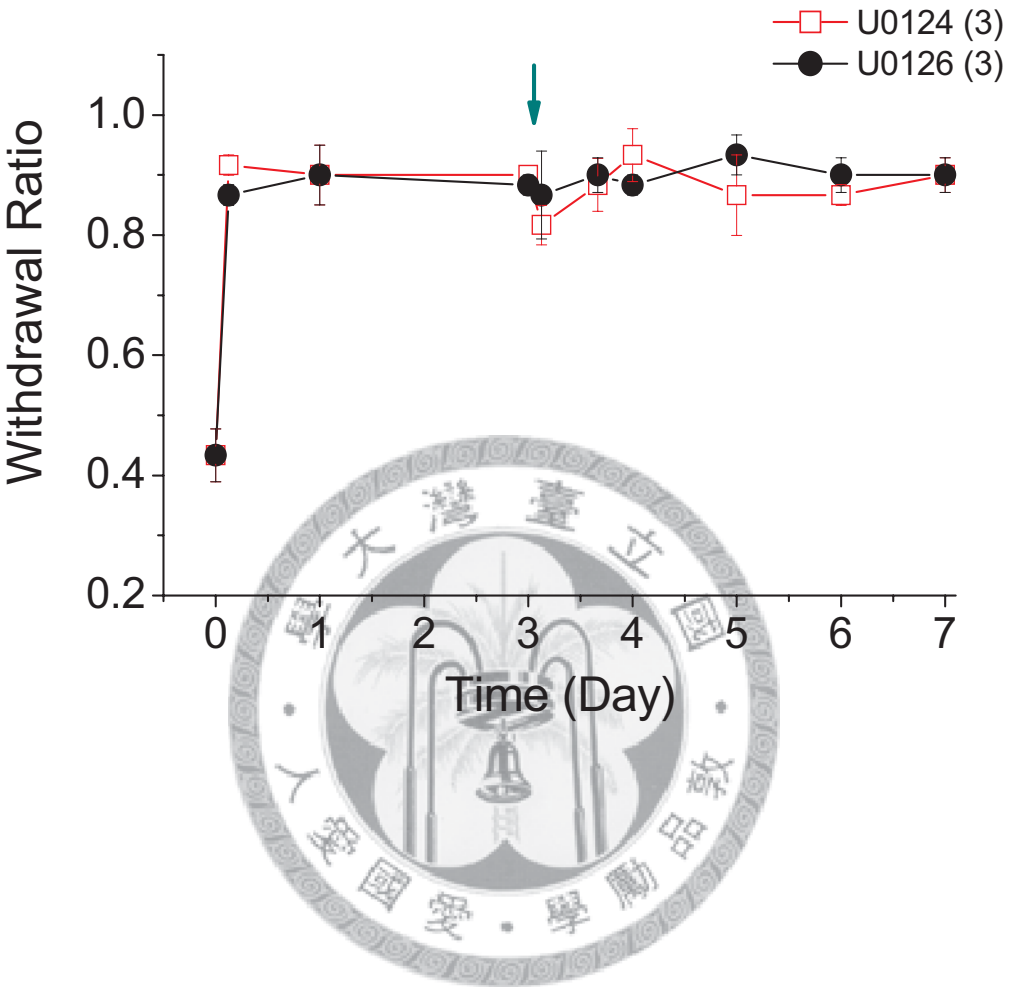

Figure 4 Infusion of U0126 in RCeA 3 days later has no effect on formalin-induced persistent mechanical hyperalgesia. Formalin induced long lasting mechanical hyperalgesia is not affected by U0126 or U0124 infusion in the right central nucleus of amygdale (RCeA) at 3 days after formalin induction. The blue arrow indicates when U0126/U0124 administrated. The treatment and behavioral test procedure is shown as P3

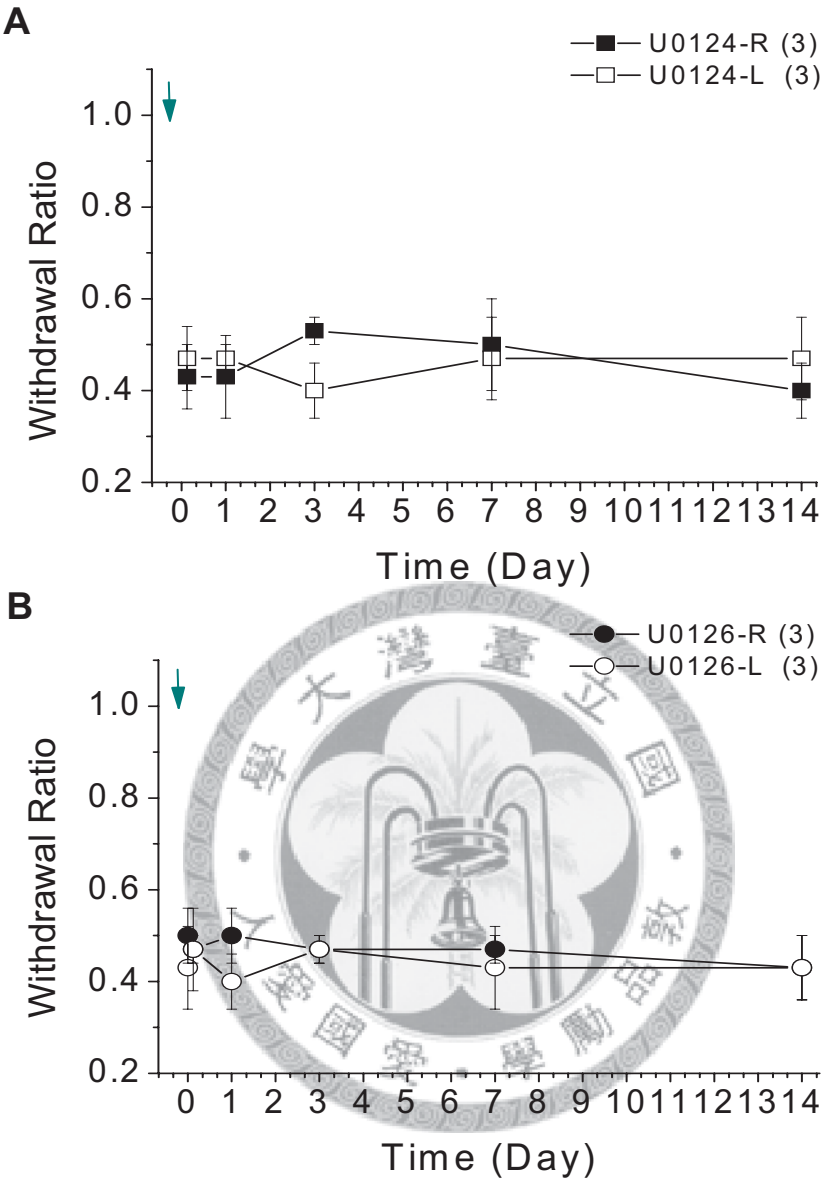

Figure 5 Intra-PVA infusion of MEK inhibitor has no effect on naïve mice. A, B. The blue arrow indicates when the acute infusion administrated. Acute intra-PVA infusion of U0124 and U0126 has no effects on withdrawal response of both hind limbs to mechanical stimuli in naïve mice. The treatment and behavioral test procedure is shown as P5.

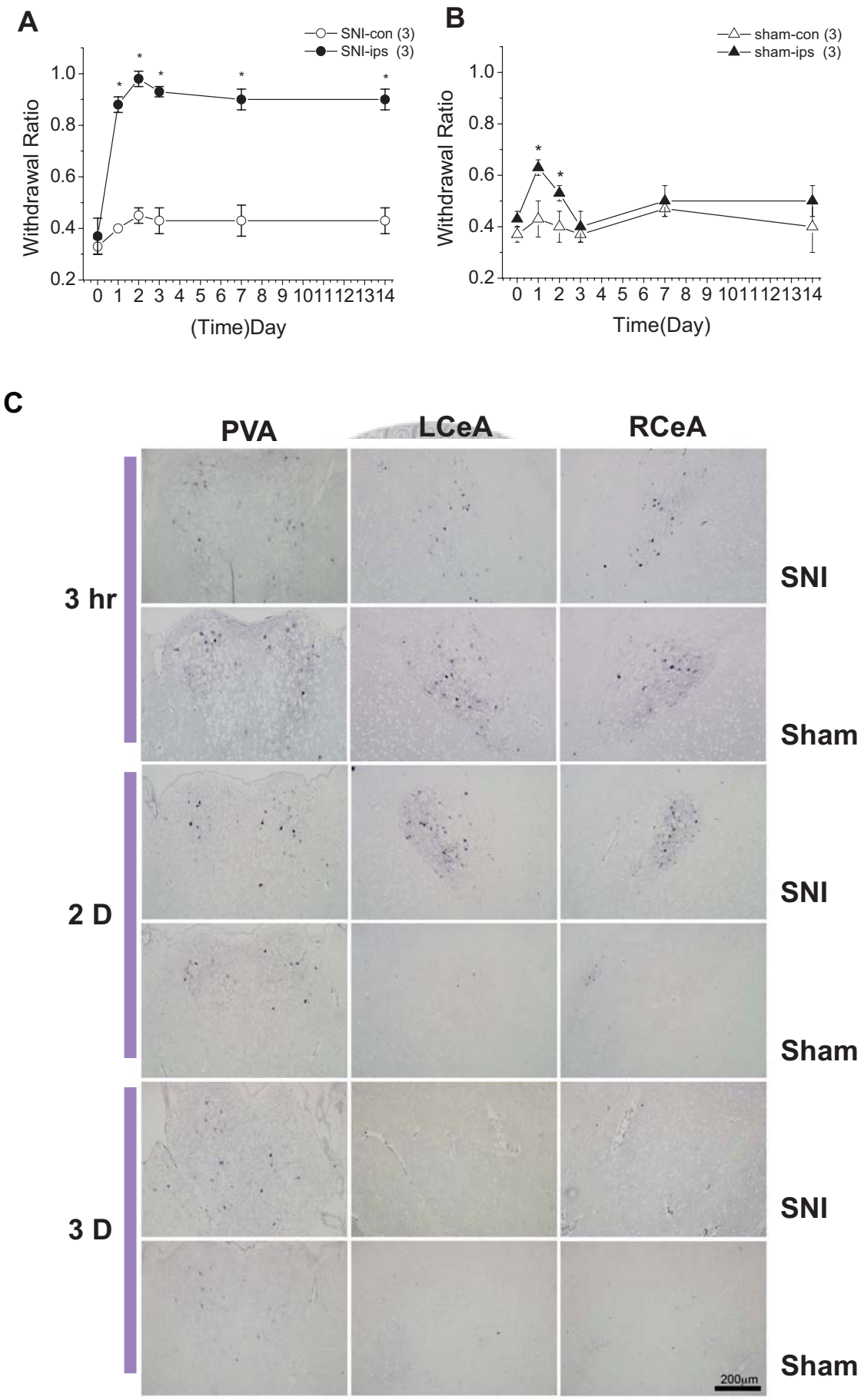
A
SNI-con (3)
SNI-ips (3)
Withdrawal Ratio
(Time)Day
B
sham-con (3)
sham-ips (3)
Withdrawal Ratio
Time(Day)
C
PVA
LCeA
RCeA
3 hr
2 D
3 D
SNI
Sham
SNI
Sham
SNI
Sham
200μm

Figure 6 Nociceptive behaviors and ERK activation in PVA and CeA in SNI model. A. SNI surgery induced unilateral chronic mechanical hyperalgesia which lasts for at least 2 weeks in mice. **B**. The SNI-sham operation induced transient mechanical hyperalgesia in the ipslateral leg. **C**. Enhanced pERK positive signals in PVA and amygdala were detected in both SNI and sham operated mice 3 hr, 2 days after surgery, the pERK signals fainted in sham operated mice in PVA and disappeared in amygdala 2 days after surgery respectively (* significantly different between groups, compared to the contralateral limb).

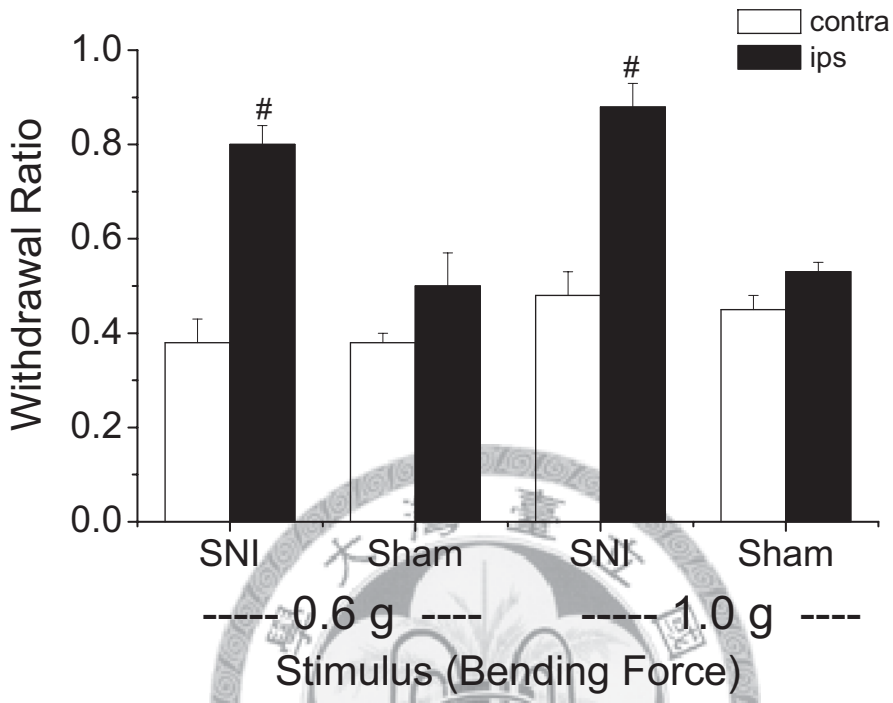

Figure 7 Mechanical hyperalgesia induced at 2 hrs after Spared Nerve Injury (SNI).. Two hours after surgery, the withdrawal ratio of the SNI-operated hind limb has an significant increase in response to 0.6-g and 1-g bending force of von Frey filament whilst the sham-operated hind limb showed no sight of increase of withdrawal ratio compared to their contralateral limbs, non-operated ones. (# $P<0.05$, pared T-test, significantly greater than the contralateral limb).

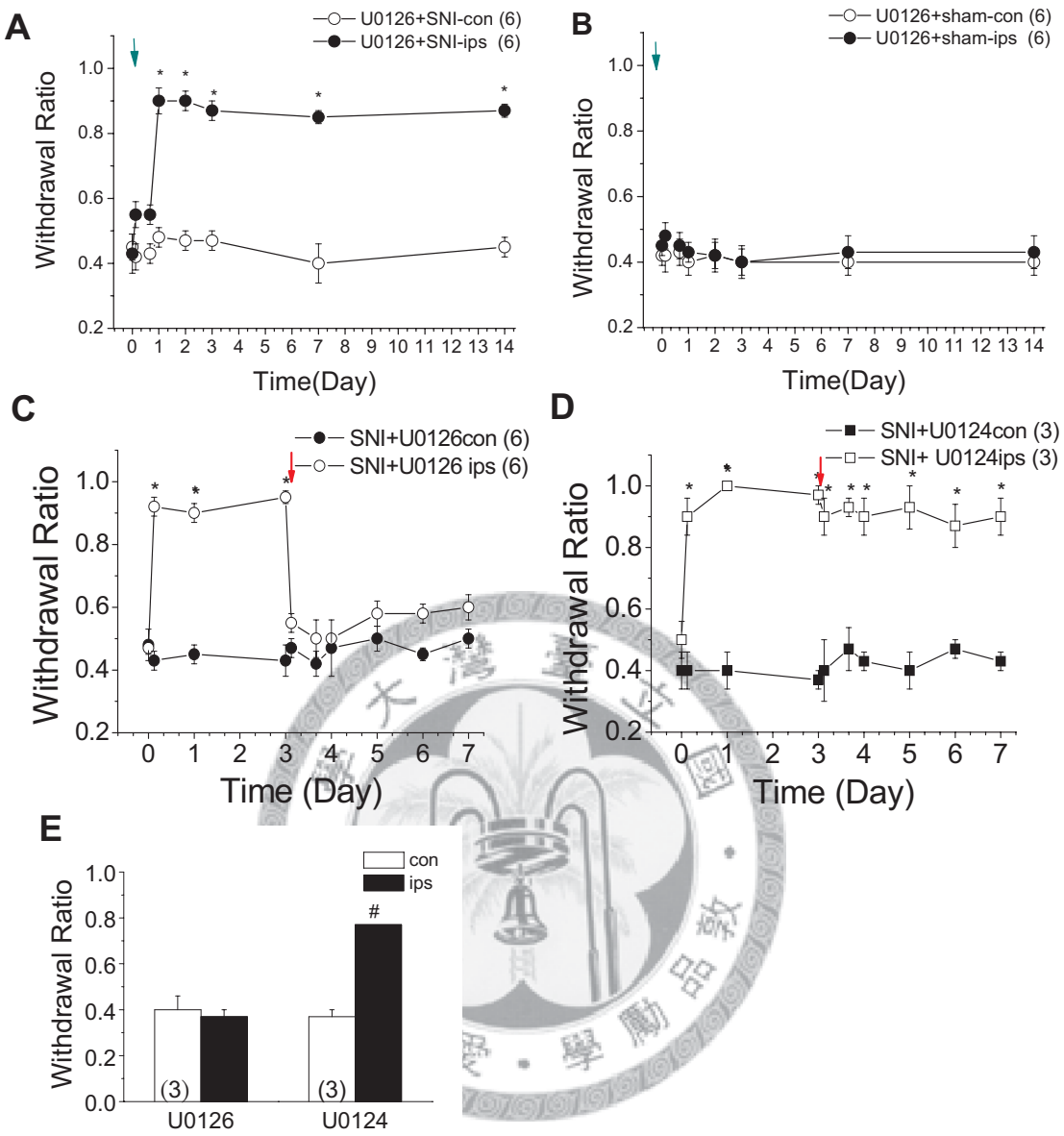

Figure 8 The effect of MEK inhibitor on SNI-induced mechanical hyperalgesia. **A, B.** U0126 intra-PVA infusion (arrow) before surgery reduced the SNI-induced mechanical hyperalgesia transiently and prevented sham operation induced mechanical hyperalgesia. **C, D** Infusion of U0126 at 3 days after SNI induction blunts the mechanical hyperalgesia whereas U0124 infusion has no effect on the withdrawal response. **E** The withdrawal ratio on Day 14 after SNI induction illustrates the inhibition effect of U0126 lasts (* significantly different between groups; # significantly greater than basal).

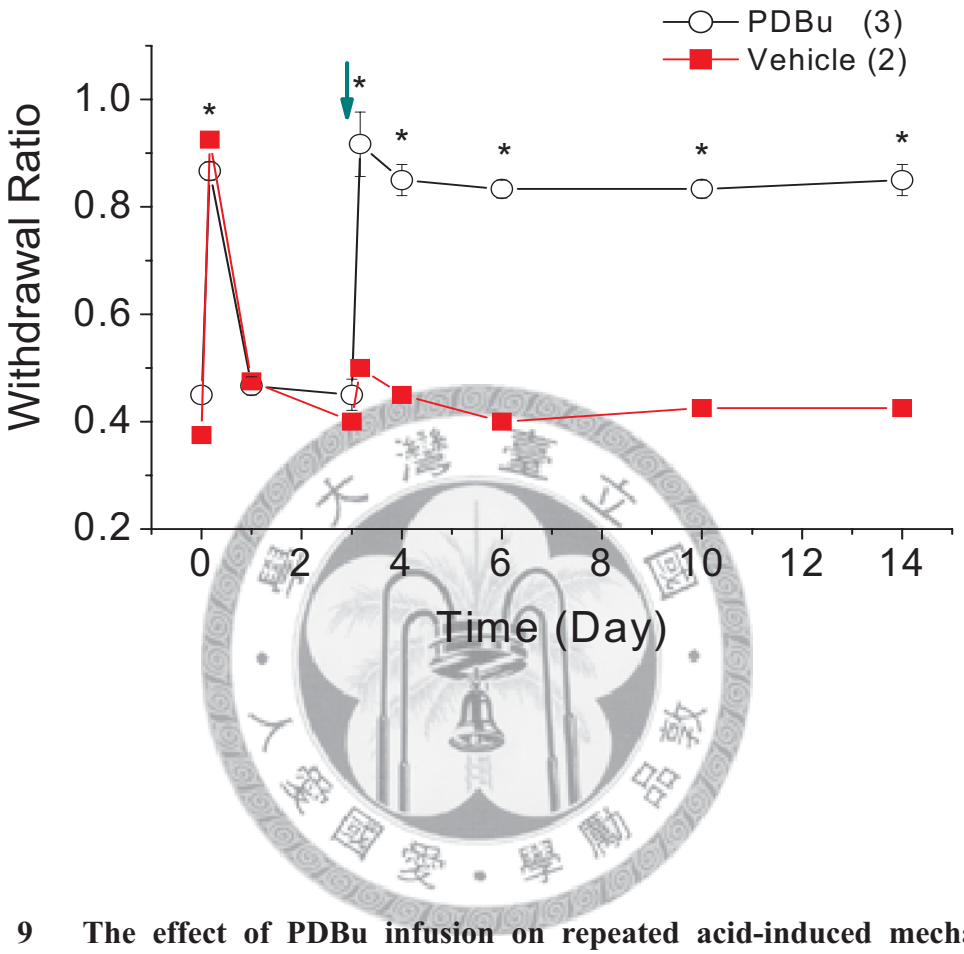

Figure 9 The effect of PDBu infusion on repeated acid-induced mechanical hyperalgesia. Infusion of PDBu, a PKC agonist, instead of the second acid injection induced the increase of the withdrawal ratio (black circles) and triggers the development of persistent mechanical hyperalgesia whereas the vehicle, 50% DMSO, has no effect on the withdrawal incident in response to 1-g von Frey monofilament (* significantly different between groups, compared to vehicle).

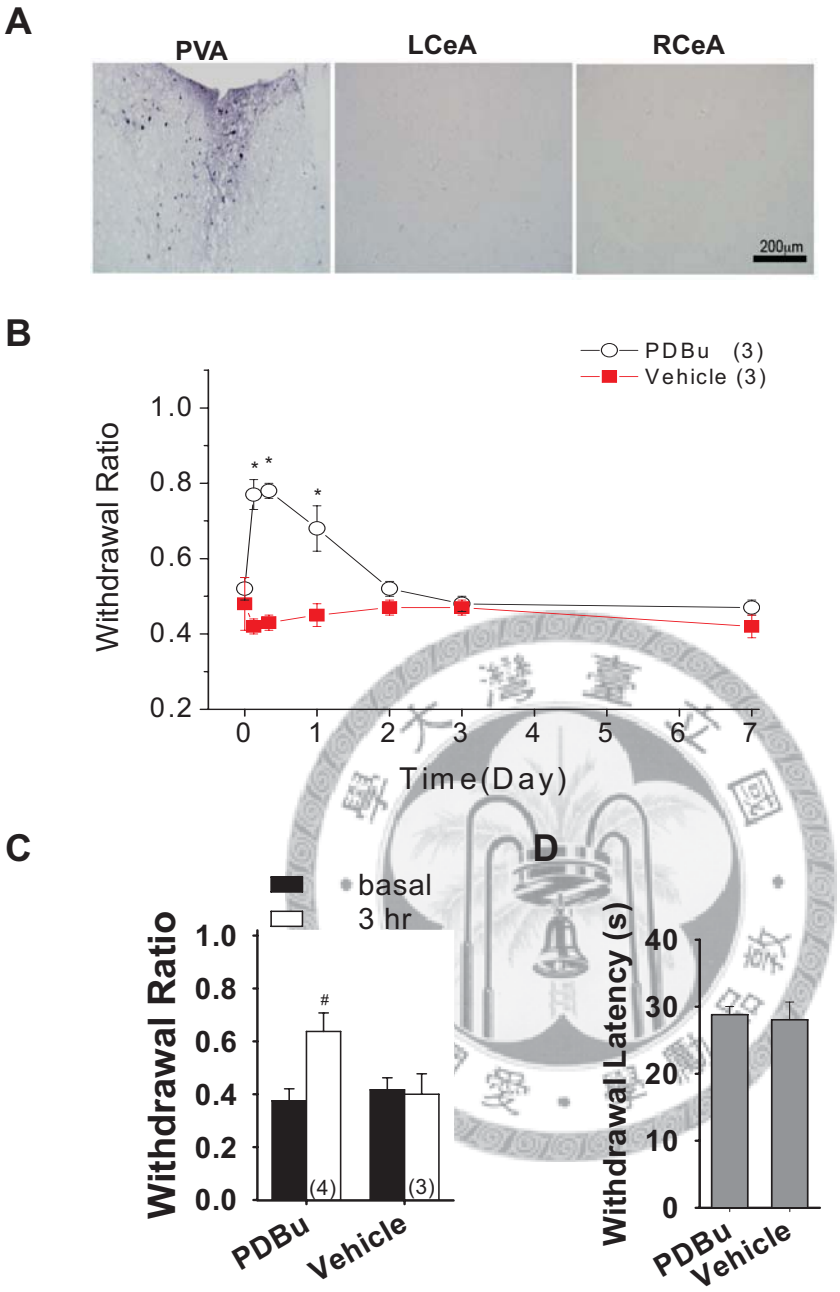

Figure 10 Intra-PVA infusion in naïve mice induced mechanical hyperalgesia. A. Intra-PVA PDBu infusion activated ERK in PVA but not amygdala. **B, C.** PDBu but not vehicle control infusion in naïve mice induced mechanical hyperalgesia transiently (open circles).. **D.** Thermal sensitivity was not changed by PDBu infusion (*significant different between groups; # significantly greater than basal).

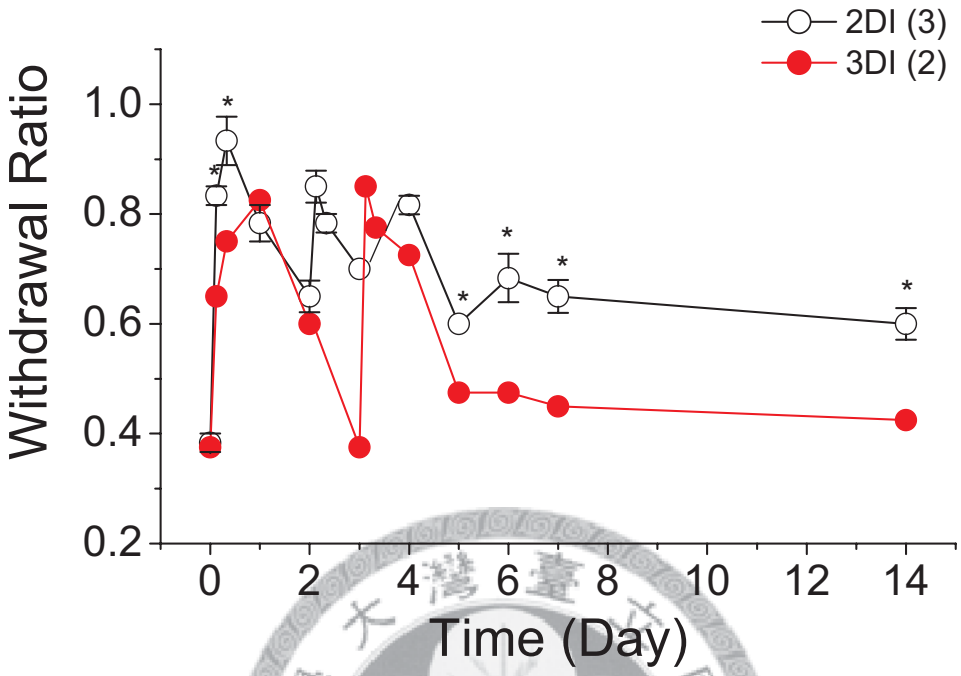

Figure 11 Repeated PDBU infusion leads to sustained mechanical hyperalgesia.

The first PDBu infusion (arrow) induced a transient increase of mechanical withdrawal ratio in each group. The mice received the second PDBu infusion 3 days apart from the first infusion (red solid circle, red arrow) displayed a second transient uproar of withdrawal ratio in response to 1-g von Frey filament. Interestingly, the mice received the second PDBu infusion 2 days apart (open circle) displayed a sustained mechanical hypersensitivity that lasted at least for one week (* significantly different from the control group).