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後端島腦皮層在大鼠神經痛模式中扮演之角色

The Role of Posterior Insular Cortex in Rat Model of Neuropathic Pain

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本論文係葉瀚元君（R00B41009）在國立臺灣大學動物學研究所完成之碩士學位論文，於民國一百零二年七月三十一日承下列考試委員審查通過及口試及格，特此證明

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終於把口試和論文都結束了！想起來覺得真的很不可思議，口試短短的九十分鐘，論文少少的四、五十頁，卻是我兩年七百三十一天的研究成果耶！蘇打綠那首「十年一刻」一直迴響在我腦海裡。回想起這兩年來，真的是經歷了不少的酸甜苦辣:剛成為小碩一還沒開學就要參加的，內容硬的要死但卻收穫豐富的summer camp；對天真活潑的大學生傳道授業解惑的普動實和動生實，得到學生肯定的感覺很棒；每天忙到半夜只好招計回家就為了神解實和神生實能做出好的結果；考驗記憶力的神經解剖學；每到聖誕節的交換禮物；物所舉辦的超歡樂梅峰遊、金山遊和桌球賽；seminar 上台前恐怖的心理壓力；為了抒發壓力的運動團。這些對我來說都是永遠而且寶貴的回憶，但也因為有很多人的幫忙，我才能快樂順利的度過碩士生涯。

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摘要

神經痛是臨床上難以根治的一種神經疾病。它會造成中樞神經系統神統神經可塑性永久的變化。島腦皮質是大腦中可接受不同種類感覺訊息傳入的部位，許多研究顯示後端島腦能接收體感覺訊息，尤其是疼痛相關訊息的處理。然而，後端島腦對於神經痛的形成與維持的貢獻仍然不清楚。本實驗將後端島腦進行永久性破壞，觀察破壞後對神經痛模式大鼠行為指標的影響。結果顯示，破壞後端島腦可以使得神經痛造成的機械性觸感痛有緩慢少許的回覆；冷覺反應也在破壞後端島腦後有短暫減緩的情形。神經痛之前先破壞後端島腦則造成機械性觸感痛發展較輕微；自發性疼痛沒有明顯差別，而冷覺觸感痛有加速發展的現象。神經追蹤劑的研究結果發現，後端島腦會投射至視丘的後端三角核，而此核區主要接受來自於脊髓的痛覺訊息。本實驗發現到後端島腦可能參與了神經痛在機械性觸感痛的長期維持。此外，不同的神經痛症狀可能由不同大腦核區處理。

關鍵字: 坐骨神經分支選擇選擇結紮切斷、永久破壞、機械性觸感痛、順向和逆向追蹤劑、視丘後端三角核

Abstract

Neuropathic pain is an intractable disease in daily life and clinical research. It can result in long-term changes in central nervous system. Insular cortex is a brain region participated in processing of different sensory modalities. Evidences have also shown that posterior insular cortex may be related to somatosensory perception especially in nociception. However, the role for how PIC contributes to the initiation or maintenance of neuropathic pain is less understood. In the present study, permanent lesion by NMDA excitotoxicity in PIC was used to assess the response to pain. Results showed that after PIC lesion in neuropathic rats, the mechanical threshold recovered gradually. The spontaneous paw lifting showed no improve, and withdrawal response to cold were transiently diminished. PIC pre-lesion resulted in less decreased mechanical threshold, and transient decrement of spontaneous paw lifting. However, there were faster development of cold allodynia. Tracer study revealed that PIC had a strong connection to posterior triangular thalamic nucleus and periaqueductal gray. These data suggested the partial role of PIC to maintain mechanical allodynia in neuropathic pain. Moreover, spontaneous pain, mechanical allodynia and cold allodynia of neuropathic pain might be differentially processed in the forebrain.

Key words: spared nerve injury; permanent lesion; mechanical allodynia; anterograde and retrograde tracer; posterior thalamic nucleus, triangular part

Introduction

Neuropathic pain, defined by International Association for Study of Pain (IASP), is a chronic painful state caused by the neuronal damage of peripheral nervous system (PNS) or central nervous system (CNS). Neuropathic pain results in (1) spontaneous pain, ongoing painful feeling without any peripheral stimulation; (2) allodynia, pain elicited by normally innocuous stimulation; (3) hyperalgesia, hyper-responsiveness to painful stimulation. These symptoms affect patients a lot in daily life. Moreover, neuropathic pain could last for a long time, even the origin of damage had cured. For now there is no adequate way to long-term diminish the suffering from these symptoms. Therefore, it is important to understand the underlying mechanism of neuropathic pain so that better treatment can be designed.

Peripheral mechanisms of neuropathic pain

Spontaneous pain is the most distinct trait in neuropathic pain. After the damage of nerve, neuroma develops and starts firing spontaneously in the injured site. On the other hand, the adjacent intact nerve also presents low spontaneous activity (Djouhri et al., 2012; Djouhri et al., 2006). Many evidences have revealed that ion channels, including voltage-gated sodium channels and voltage-gated calcium channels, contribute to the generation of spontaneous activity (Berta et al., 2008; Hendrich et al., 2008; Li et al., 2004; Nassar et al., 2006).

Another characteristic of neuropathic pain is allodynia, which means gentle tactile stimulation could induce pain feeling. In normal condition, peripheral Aβ fibers relay tactile information. However, after nerve injury, Aβ fibers somehow receive nociceptive information normally the Aδ and C fibers mediate. The sprouting of Aβ fibers from lamina III-V to lamina I can be observed, and the expression of pain-related immediate early gene, *c-fos,* is induced by tactile stimulation after neuropathic pain (Bester et al., 2000). The activation of postsynaptic pathway from Aβ fibers to lamina I also lead to mechanical allodynia (Sandkuhler, 2009). The expression of transient receptor potential (TRP) receptor, including TRPV1, TRPA1 and TRPM8, increases in DRG after nerve injury, and this causes the thermal hyperalgesia and cold allodynia (Xing et al., 2007).

Disinhibition is also observed after nerve injury. In the spinal cord, the inhibitory GABAergic and glycinergic neurons decrease after nerve injury, leading to the over-excitation of nociceptive postsynaptic neuron and the increasing inputs of Aβ fibers to lamina I (Baba et al., 2003). In addition, the unbalance of descending modulation is also involved in this mechanism (Leong et al., 2011).

Central sensitization

Central sensitization is long-term facilitation of central nervous system. At the spinal cord level, the glutamate NMDA receptors is phosphorylated, and the trafficking of NMDA and AMPA receptors to synapse are also increased in neuropathic pain

(Ultenius et al., 2006). Central sensitization also happens at the supraspinal level, and it causes long-term changes in several brain regions. Owing to the improvement of brain image technology including functional magnetic resonance image (fMRI) and positron emission tomography (PET), we can easily detect whole brain activity of human or animals in neuropathic pain.

According to image research, numerous brain regions which process and perceive the pain stimulation cooperate as a pain matrix. This pain matrix can be simply divided into lateral pain system and medial pain system. Lateral pain system is involved in the sensory-discriminative aspect of pain, which precisely reports the spatial distribution and the intensity of pain sources. Primary and secondary somatosensory cortex (S1 and S2), ventral posterior thalamic nucleus (VP) are included in lateral pain system. After nerve injury, the thalamic firing rate is changed to more activated, and the firing pattern gets more irregular (Iwata et al., 2011; Saab, 2012). SI also shows abnormal high activity and alteration of dendritic spines in neuropathic pain animals (Kim and Nabekura, 2011; Quiton et al., 2010).

Medial pain system participates in affective-emotional aspect of pain, which is related to the feeling of pain stimulation. Medial thalamus, amygdala, anterior cingulate cortex (ACC) and prefrontal cortex (PFC) are believed to encode the unpleasantness of pain. ACC is the region which gets more attention on studying neuropathic pain. Lesion

of rostral ACC could not alleviate tactile and thermal allodynia in neuropathic pain, but it disrupts animal's conditioned preference to analgesic-paired chamber, indicating that ACC is something to do with the emotional component of pain (Qu et al., 2011). After nerve injury, the NR2B subunit of NMDA receptor are up-regulated, resulting of long-term amplification of local ACC circuit (Xu et al., 2008). The morphology of pyramidal cells in ACC and medial prefrontal cortex also changes after nerve injury (Metz et al., 2009).

Posterior insular cortex and neuropathic pain

Insular cortex is located under the parietal lobe and temporal lobe in primate brain, or around the rhinal fissure of rat brain. Insular cortex can be partitioned into anterior insular cortex (AIC) and posterior insular cortex according to physiological and anatomical evidences. Anterior and posterior insular cortices both contribute to pain processing in different ways. Many research have revealed that AIC is involved in emotional aspect and the descending modulation of pain. However, the role of PIC is still less understood and controversial.

PIC has been regarded as multisensory perceiving region. This area responses to different sensory modalities, including gustatory, visceral, auditory and somatosensory stimulation. Moreover, Single neuron in PIC could response to more than one kind of stimulation (Hanamori et al., 1998a, b). Neurons in PIC are also activated under painful

stimulation both in animal and human studies (Garcia-Larrea, 2012; Garcia-Larrea et al., 2010; Isnard et al., 2011). In patient suffering from PIC epilepsy, they often report the painful feeling during seizure. Direct stimulation of insular cortex can also induce pain (Mazzola et al., 2009; Ostrowsky et al., 2002). In patient with PIC lesion, higher pain rating to thermal noxious stimulation occurs (Starr et al., 2009). In addition, there are somatotopic organization in rodent and human PIC (Baumgartner et al., 2010; Benison et al., 2007; Brooks et al., 2005; Rodgers et al., 2008). In functional connectivity studies, PIC is more likely to connect with S1 and motor cortex, indicating that PIC could participate in lateral pain system and function as multisensory integration (Cauda et al., 2011; Peltz et al., 2011).

Although PIC has lots to do with nociception, the understanding of the relation to neuropathic pain is still limited. Benison et al. reported that caudal granular insular cortex (CGIC) was necessary for the maintenance of mechanical allodynia in rats with neuropathic pain (Benison et al., 2011). However, the effects of PIC on other neuropathic symptoms, including spontaneous pain, hyperalgesia and temperature-evoked allodynia, are still unknown.

Neuronal connection of PIC

Insular cortex has numerous cortical and subcortical areas, revealing that insular cortex is involved in many sensory modalities. Insular cortex can be briefly divided into

three parts according to the connection and positions. The anterior insular cortex, so-called prefrontal insular cortex by Guldin et al. (1983), locates at anterior one-thirds of whole insula and has connection to mediodorsal thalamic nucleus (MD), medial prefrontal cortex (mPFC) including prelimbic and infralimbic cortex (PrL and IL) . These brain regions are believed to function as cognitive and emotional processing. In addition, the most anterior insula called rostral agranular insular cortex (RAIC) robustly communicates with ACC, orbitofrontal cortex, amygdala, and medial thalamus including MD, centrolateral thalamic nucleus and nucleus submedius (Sm), indicating that RAIC is involved in the medial pain pathway (Jasmin et al., 2004) . Connections with rostroventral medulla (RVM) and periaqueductal gray (PAG) reveals that RAIC also participates in descending pain modulation. The middle part of insular cortex engages in gustatory and visceral functions. This region connects with parvicellular part of ventroposterior medial and ventroposterior lateral thalamic nucleus (VPMpc and VPLpc) and IL (Allen et al., 1991; Saper, 1982; Shi and Cassell, 1998) .

The posterior insular cortex is also called associative insular cortex because this region has been reported involving in the processing different sensory inputs. This region has robust interconnection with posterior thalamus, especially the posterior triangular thalamic nucleus (PoT), which is located at the caudal part of posterior thalamic nucleus (Po). PoT has been reported receiving the projection of spinothalamic

tract from superficial laminae of spinal cord (Al-Khater and Todd, 2009; Gauriau and Bernard, 2004a; Zhang and Giesler, 2005). PIC also communicates with primary and secondary somatosensory cortices, indicating the function of PIC as pain and tactile processing. The projections to spinal trigeminal nucleus, lateral PAG and RVM reveal that PIC could participates in pain modulation. In addition, PIC also has connections with VPMpc, VPLpc and medial geniculate nucleus (MGN). It can be deduced that PIC may be a integrated region of different sensory modalities.

Appropriate animal model for investigating neuropathic pain

There are many animal models for preclinical research of neuropathic pain. Many of these animal model focus on lumbar plexus, including L4~L5 spinal nerve, sciatic nerve trunk and its branches. Spinal nerve ligation (SNL) is tight ligation of L5/L6 spinal nerve. SNL produce obvious neuropathic pain-like behaviors including spontaneous pain and evoked pain (Kim and Chung, 1992). In chronic constriction injury of sciatic nerve, four chromic catgut loosely ligate the sciatic nerve trunk, and it develops mechanical/thermal hyperalgesia and allodynia (Bennett and Xie, 1988).

Spared nerve injury (SNI) is induced by tight ligation and transection of two branches of the sciatic nerve. Animals preformed SNI show robust hypersensitivity in mechanical, thermal and cold stimulation, and it takes advantages of short-term induction and long-term maintenance (Decosterd and Woolf, 2000; Richner et al., 2011).

Literatures have suggested the spinal and supraspinal mechanism contributing to neuropathic pain after SNI (Suter et al., 2003; Xie et al., 2005). In this manner, this animal model is suitable and chosen to be the animal model of neuropathic pain in the present study.

Hypothesis

Nerve injury can cause neuropathic pain, which results in central sensitization of the brain. On the other hand, evidences suggest that PIC is involved a lot in pain processing and perception. We hypothesize that sensitization of PIC after nerve injury contributes to neuropathic pain.

Aim of this study

(1) the neuronal tracing was also used to confirm the connection between PIC and other brain regions which participate in pain perception.

(2) Permanent lesion of PIC by NMDA excitotoxicity was used to assess the contribution of PIC to neuropathic pain.

Materials and methods

Animals

Adult female Sprague-Dawley (SD) rats were obtained from BIOLASCO (Taipei, Taiwan) and housed in animal room of Life Science building, National Taiwan University. Animals lived at an environment of 12h light/dark cycle and at 22℃. Food and water were available *ad libitum*. The rats aged 9-10 weeks and weighed 230~300 g were allowed to use in any experiments. All animal cares and experimental procedures were approved by the Institutional Animal Care and Use Committee, National Taiwan University. This study was in accordance with the "Codes for Experimental Use of Animals" of the Council of Agriculture of Taiwan based on the Animal Protection Law of Taiwan.

Experiment 1. PIC lesion study

Spared nerve injury (SNI)

Animals were anesthetized by ketamine and xylazine. The surgery was performed as Decosterd et al. (2000). Briefly, the fur of left thigh was shave, skin was incised, and the left sciatic nerve was exposed. Common peroneal nerve and tibial nerve were tightly ligated by 6/0 silk, and a fragment of two nerves were cut by corneal spring scissors. Sural nerve was left intact. The muscle was sutured by 6/0 silk and the skin was sutured

by 4/0 silk. To prevent infection, lincomycin hydrochloride (30 mg/kg) was administrated into right gastrocnemius muscle to prevent infection.

Behavioral tests

All the following tests were conducted during daytime (9:00~18:00).

(1) mechanical allodynia. Each rat was placed in a acrylic test box for habituation 5~7 days before tests. On the test day and after habituation of 5-10 min, a series of von Frey hairs (0.4 g, 0.6 g, 1 g, 2 g, 4 g, 6 g, 8 g, 15 g, Touch Test Sensory Evaluators, North Coast) were used to stimulate the outer plantar surface of both hindpaws vertically. Stimulating started at 2 g with the duration of 5~8 sec. Whenever paw withdrawal response occurred during stimulation, the next lighter von Frey was applied. Whenever no withdrawal response occurred, the next heavier one was applied. Three repeats were applied with intervals over 1 min. The 50% withdrawal threshold was calculated (Chaplan et al., 1994).

(2) spontaneous pain. Each rat was placed in a 28 cm × 28 cm × 40 cm transparent acrylic chamber on a 30℃ hot plate (Model 390, IITC Life Science). After habituation of 5 min, the spontaneous lifting of both hindpaws was counted in 30 min. Paw lifting with locomotion or body reposition were excluded.

(3) Cold allodynia. Acetone-induced cold allodynia test was used (Choi et al., 1994). Animals were habituated and tested in test box as mechanical allodynia. Acetone

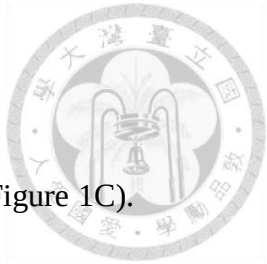
was dropped on both hindpaws in turn, and the duration of paw withdrawal within 1 min was counted with a 20 sec cut-off. Five repeats were applied with 5-min intervals.

Chronic lesion of PIC

Rats were anesthetized by sodium pentobaribital (50 mg/kg, i.p.) then fixed on stereotaxis apparatus (David Koft Instrument). The body temperature was maintained at 37℃ by heat plate with anus sensor. Craniotomy bilaterally was performed to expose the brain surface vertical to PIC and the dura matter was removed. A 10 μL Hamilton syringe with a glass pipette (tip size ~25 μm, # 602500, A-M Systems) sealed by silicon was slowly inserted into PIC and left *in situ* for 10 min (coordinates: -1.00 and -2.00 mm from bregma for anteroposterior axis, 6.00 mm for mediolateral axis, 4.80 mm for dorsoventral axis). Each site was injected 5% NMDA (Sigma) solution in 0.01 M PBS of 0.3~0.5 μL by hand bilaterally. Sham lesion was the injection of 0.3~0.5 μL PBS. After surgery, the incision was sutured and lincomycin hydrochloride was administrated.

Experimental procedures

In order to investigate the effects of PIC lesion on neuropathic pain, three experimental procedures were conducted: (1) in the post-lesion group, rats were performed SNI surgery and then PIC lesion after 2 weeks (Figure 1A). (2) in pre-lesion group, rats were performed PIC lesion and after 2 weeks the SNI surgery was applied



(Figure 1B). (3) in lesion-only group, rats were performed PIC lesion only (Figure 1C). Behavioral tests were carried out every 7 days in all three experiments. The total testing periods were 7 weeks in post-lesion and pre-lesion group and 5 weeks in lesion-only group.

Histology

Rats were deeply anesthetized by sodium pentobaribital and perfused transcardially by saline with 0.5% sodium nitrite and tri-sodium citrate then by 4% paraformaldehyde in 0.1M PBS. The brain was removed and post-fixed in paraformaldehyde for 1~3 days then changed to 0.1M PB with 30% sucrose until the brain was sunk. Brain was frozen-sectioned at 50 μm and collected floating. Slices were treated with 3% H_2O_2 solution, washed three times for 10 min in 0.3% TPBS, and treated with blocking solution including 2% bovine serum albumin (BSA), 10% normal goat serum (NGS) in TPBS. Slices was incubated with mouse anti-NeuN (MAB377B, Chemicon) or anti-GFAP (MAB360, Chemicon) at 4℃ for 48 h in buffer consisting of 0.1% BSA in TPBS. Slices were kept incubating at room temperature for 1 h, washed in TPBS for three time, then incubated with biotinylated goat-anti mouse IgG at room temperature for 1 h. Slices were then washed three time for 10 min in TPBS, incubated with Avidin/Biotin complex (ABC, PK-4000, Vector laboratories) solution for 1 h. After three washes in TPBS, slices were treated with 3,3'-diaminobenzidine (DAB) and

visualized with 3% H_2O_2 solution. Slices were mounted on slide, air dried, dehydrated by graded descending ethanol, cleared by Hemo-De and coverslipped. With the purpose of verification of lesion extent, Nissl stain was also performed. For Nissl stain, mounted and air dried slides were rehydrated by descending alcohol (100%, 95% and 70%) and ddH_2O, stained in cresyl violet, rehydrated by ascending alcohol (70%, 95% and 100%), and then cleared and coverslipped. The lesion extent of PIC was examined under light microscope and confirmed by rat brain atlas (Paxions and Watson, 1988). Slides were examined under light microscope.

Data analysis

For all of the three behaviors, the change of withdrawal threshold or paw lifting through time was plotted. In post-lesion group, changes of withdrawal threshold responded to mechanical stimulation was presented by percentage related to D7. All the data in illustrations are presented by mean ± SEM. The lesion effect was tested by two-way repeat measurement ANOVA. One factor includes lesion groups and both hindpaws (PIC lesion ipsilateral, PIC lesion contralateral, sham lesion ipsilateral, and sham lesion contralateral), and the other factor is time before and after SNI surgery. Least significant difference (LSD) was chosen to be the *post hoc* test. All the statistics are done by SigmaPlot.

The analysis of PIC lesion was presented with the largest and the smallest extents, identified by the rat brain atlas (Paxinos and Watson, 2007). Lesion was defined as extents including neuronal loss, tissue necrosis and astrogliosis (including scar formation and over-proliferation of astrocytes) observed by Nissl stain and immunohistochemistry of NeuN and GFAP.

Experiment 2. Neuronal connectivity of PIC

Tracer injection

To investigate the interconnection of PIC and other brain regions, 2% biotinylated dextran amine (BDA, MW 10,000 and MW 3,000, Molecular Probes) dissolved in ddH_2O was injected. Animals were anesthetized using a mixture of ketamine (75 mg/kg) and xylazine (15 mg/kg) then fixed on stereotaxis apparatus. The experimental procedure was the same as PIC lesion (Coordinates of injection site: Anteroposterior axis: -1.50 to -2.00 mm from bregma; mediolateral axis: 6.0 mm; dorsoventral axis: 4.8 mm). Larger extent (1.0~1.5 μL) or smaller extent (0.2~0.5 μL) of BDA was injected by hand then kept in brain for 10 min. After surgery, the incision was sutured and lincomycin hydrochloride was administrated.

Histology

Rats were allowed to recover and survive for 10 days. After that, rats were perfused, post-fixed and frozen-sectioned the same as lesion study. Slices were washed three

times for 5 min in PB, treated with ABC kit, washed five times for 10 min, treated with DAB and then visualized by 3% H_2O_2 solution. Slices were mounted on gelatin-coted slide, air dried, dehydrated in ascending alcohols, cleared by Hemo-De, and coverslipped. Another set of slices were counterstained by Nissl stain. Labeled terminal buttons and cell bodies were photographed, and the labeled regions were identified by the rat brain atlas.

Results

Experiment 1. PIC lesion study

1.1 Behavioral effects of SNI

On D7 and D14 after SNI surgery, the withdrawal threshold responding to mechanical stimulation of injury (ipsilateral) site was significantly dropped compared to D0 ($p<0.001$, Figure 2A). For spontaneous pain, the lifting times of ipsilateral hindpaw site on D7 and D14 were significantly more than D0 and contralateral site ($p<0.001$, Figure 2B). In cold stimulation, the withdrawal duration of ipsilateral was higher in D7 and D14 than Day 0 and contralateral site ($p<0.001$, Figure 2C). In contrast, the intact (contralateral) hindpaw showed unchanged before and after SNI in all of three tests. These data indicate that SNI surgery results in the hypersensitivity of ipsilateral hindpaw to either evoked and spontaneous pain, and it also validates the following lesion tests.

1.2 Histological verification of PIC lesion

The extent of PIC lesion was confirmed by Nissl stain and immunohistochemistry. In post-lesion group, reconstruction of the largest and the smallest lesion extent was shown (Figure 3A). The largest extent covered PIC from Bregma 0.00 mm to +3.00 mm, expanding to part of S2 and striatum. The smallest extent included PIC from -0.48 mm to -2.04 mm. Nissl stain showed cell necrosis near the injection site and neuron loss

around (Figure 3C). To confirm the exact lesion range, NeuN and GFAP immunostaining were preformed. Histology showed no NeuN labeled signals in lesion site, and there were severe gliosis around the cell necrosis. On the contrary, there was no damage in PIC in sham lesion group.

In pre-lesion group, NMDA also caused severe destruction in PIC including adjacent small part of S2 and striatum. The smallest lesion extent was in deep layer of PIC, from -1.44 mm to -3.00 mm (Figure 5A). Nissl stain and immunohistochemistry also showed neuron loss and gliosis in lesion site (Figure 5C). Sham lesion also showed no tissue damage.

In only-lesion group, the largest lesion extent covered the whole PIC and SII, even the brain region posterior to PIC. The smallest lesion extent of left hemisphere was only in PIC, from -0.48 to -3.00, while there was no lesion in right hemisphere. Like other two groups, PIC lesion resulted in tissue damage in verification by Nissl stain and immunohistochemistry (Figure 7C).

1.3 post-lesion group

In this group, after PIC lesion, the threshold to mechanical stimulation of ipsilateral site gradually increased and get significance from D28 to D48 than that of D7 ($p<0.05$ on D28 and D35; $p<0.001$ on D42 and D49). In comparison with sham lesion group, withdrawal threshold also showed significance ($p<0.001$, Figure 4A). In

spontaneous pain test, the ipsilateral site of PIC lesion group showed unchanged in lifting times before and after PIC lesion, whereas the lifting times of sham lesion group increased gradually (Figure 4B). There were no differences between lesion and sham group. In cold stimulation, PIC lesion did not change the withdrawal duration of ipsilateral hindpaw in the whole periods. It also showed no differences compared to sham group except D21. In all of three tests, PIC lesion had no effect on contralateral hindpaw through time or compared to sham group.

1.4 pre-lesion group

After PIC lesion before SNI, there were no differences between PIC lesion and sham lesion in three behavioral tests. However, after SNI, the withdrawal threshold responding to mechanical stimulation in PIC lesion group decreased in a less extent than sham lesion group on D14 and D21 ($p<0.01$, Figure 6A). The spontaneous paw lifting showed no differences except D14 (Figure 6B). However, responses to acetone developed higher and faster development than sham lesion (Figure 6C). Lesion also had no effect on contralateral site in all three tests.

1.5 only-lesion group

In lesion only group, for mechanical tests and spontaneous pain, no changes on both hnidpaws after NMDA lesion (Figure 8A and 8B). There were also no significant differences between NMDA lesion and sham lesion group. However, PIC lesion caused

a significant increased duration of left hindpaw, comparing to right hindpaw, but not to sham lesion (Figure 8C).

Experiment 2. Neuronal connectivity of PIC

In order to understand whether PIC has connection to other brain regions which participate in pain processing, anterograde and retrograde tracer was injected into PIC.

2.1 Anterograde tracing

For the injection of large extent, the injection sites included whole PIC, and there are some leakage to striatum (CPu) and DEnt (Figure 9C, 10A). At this level, labeled axon terminal buttons were at CPu and central nucleus of amygdala (CeA). The contralateral PIC, CPU and CeA were also has terminals. From the most anterior (Bregma 3.72 mm), a number of labeled terminal buttons could be seen at AIC and primary motor cortex (M1), whereas there were buttons of less extent at medial prefrontal cortex, including Cg1, prelimbic cortex (PrL), and infralimbic cortex (IL, Figure 9A). Getting more posterior, labeled buttons were also located at AIC and M1. Labeling at midline was only in DP (Figure 9B). At the level of -3.00 mm, buttons were located at mediodorsal thalamic nucleus (MD), centromedial nucleus of medial thalamus, and posterior thalamic nucleus (Po). In cortex, there were obvious terminals at bilateral perirhinal cortex (PRh), dorsolateral entorhinal cortex (DLEnt, Figure 9D). The bilateral basolateral amygdala (BLA) also showed labeled buttons. At bregma -4.20

mm, labeled buttons were located at ipsilateral PRh and bilateral DLEnt with ipsilateral dominant (Figure 9E). In thalamus, numerous cells in VPM were labeled (Figure 9E and 10C). More posteriorly, labeled buttons were in DLEnt and amygdalopiriform transition area (APir) of cortex (Figure 9F and 9G). Obvious and abundant buttons were observed at PoT ad PAG (Figure 10F and 10G). Labeled terminals in PAG were at lateral site, extending to -7.08 mm. Buttons in dorsal part of dorsal raphe nucleus (DRD) were also observed.

For the injection of smaller extent, no terminals were labeled (Figure 12A), but there were a few labeled cell bodies at PoT (data not shown).

2.2 Retrograde tracing

For injection site of larger extent, most tracer were injected in PIC with the center at Bregma -2.28 mm (Figure 11A). There were small leakage to the adjacent CPu. At this level, some small cell bodies could be observed at BLA. AT the level of -4.20 mm, lots of labeled cell bodies were located at VPM and VPpc (Figure 11B). There were also some cells at subthalamic nucleus (STh). More posteriorly, numerous labeled cells were found at PoT at the level of -5.64 (Figure 11C). Some terminals were observed at substantia nigra (SN). There were no labeled cells at the levels anterior to injection site.

For injection site of smaller extent, the injection was limited in the granular and dysgranular layer of PIC at the level of Bregma -2.52 mm. Labeled cell bodies were located only at PoT (Figure 12C).

Discussion

In the present study, we found that PIC lesion could attenuate the mechanical allodynia partially and gradually after nerve injury, rather than effecting the spontaneous pain and cold allodynia. Second, PIC lesion itself did not cause any neuropathic pain-like behavior. Third, studies on neuronal connection of PIC revealed that a reciprocal connection of lateral thalamus, and anterograde connection to limbic cortices and pain-modulating regions.

In post-lesion group, the mechanical threshold had a slight and gradual increment after PIC lesion. On the other hand, nerve injury in pre-lesion group caused mechanical allodynia in a less extent. These data indicate that PIC is partially engaged in the touch-evoked pain after nerve injury. Previous studies suggested that lesion of CGIC gradually reversed the allodynic behavior in chronic constriction injury, with a reverse period of ~21 days (Benison et al., 2011). This showed that CGIC may function as maintaining the mechanical allodynia, and our present data are consistent with this study.

However, there are still some behavioral differences in the present study. The reversal period prolongs to almost 30 days, and the capability of recovery were only 1 to 2 g, quite different than almost 5 g for lesion of CGIC. We found that the variation of mechanical threshold was also too high to reach statistical significance. This may due to

the different lesion range, which covered all three layers of PIC instead of CGIC only in our experiment. Insular cortex has an cortico-cortical projection rostrocaudally through granular and dysgranular layer to anterior agranular layer, S1 and prefrontal cortex (Adachi et al., 2013; Fujita et al., 2010). These brain regions are believed to participate in further modulation or descending inhibition of pain. Thus, lesion of agranular layer may block the transmission of this modulation so that it resulted in a limited and delayed recovery. Another reason may be that in some cases lesion invaded to S2 and striatum, even some part of S1. However, there were no sensory loss or impairment of body movement, indicating the minor effect of exceeded lesion range. The other reason is that, unlike sciatic nerve CCI, SNI causes a robust neuropathic pain-like behavior and lasts more than two months (Decosterd and Woolf, 2000). The effect of central sensitization may be so strong that the compensation of other brain regions are involved, thus the block of PIC only is not enough to completely recover.

Besides the mechanical allodynia, we also found that PIC lesion resulted in a delayed deterioration of cold allodynia after nerve injury. However, PIC lesion caused faster development of cold allodynia in pre-lesion group. Previous evidence had revealed that PIC receive the projection of nociceptive non-specific (NNS) neurons in PoT (Gauriau and Bernard, 2004b). NNS responds to both innocuous and noxious heat and tactile stimulation. In human studies, PIC lesion causes higher pain rating to cold

and heat stimulation. Human studies also show that PIC responds to innocuous and noxious cold and that medial pain system is dominant in cold allodynia (Seifert and Maihofner, 2007). This implies that there are cold- or heat- perceiving neurons in PIC in normal condition. After nerve injury, processing of cold allodynia may be shift to medial pain system.

PIC lesion had little effect on spontaneous pain caused by nerve injury. Spontaneous pain is a special symptom in neuropathic pain. Djouhri et al. (2006, 2012) have indicated that spontaneous pain is resulted from spontaneous firing of injured site and the intact adjacent nerve. At the supraspinal level, medial prefrontal cortex activity is involved in the emotional aspect on spontaneous pain (Apkarian et al., 2011; Baliki et al., 2006). Therefore, emotional effect of spontaneous pain may be dominant at supraspinal level. PIC is thought to be one part of sensory-aspect of pain, leading to minor change in spontaneous pain. However, pre-lesion of PIC caused a transient decrement of spontaneous paw lifting. It is likely that PIC results in the rearrangement of supraspinal structures, especially the medial pain pathway.

PIC lesion without nerve injury did not alter the threshold to mechanical stimulation. The slight increment of ipsilateral hindpaw to acetone stimulation in both PIC lesion and sham lesion group may result from the nervousness of rats. This indicates that in normal condition, PIC only has little contribution to somatosensory

processing. After neuropathic pain, PIC is sensitized and takes part in the maintenance of neuropathic pain.

In the present tracer study, PIC had a strong connection to lateral thalamus, including VP, Po and PoT. VP and PoT, especially, have reciprocal connection to PIC. Labeled areas in VP and Po may be caused by the leakage of tracer. However, there is still a potential role of PIC to modulate the sensory relay.

Another robust connection is between PIC and PoT. PoT have been extensively reported that it receive projection from superficial laminae (layer I/II) and deep lamina (layer V) from spinal cord through spinothalamic tract. Electrophysiological studies have also shown that tactile- and pinch-positive cells in PoT project to granular layer of PIC. This robust PoT-PIC connection may be a special thalamocortical pathway to process the imformation of pain. PIC also has strong projection to lateral PAG and dorsal part of dorsal raphe. This data is consistent with most recent evidence which mentioned PIC has connection to PAG, rostroventral medulla (RVM) (Sato et al., 2013). PAG, dorsal raphe and RVM are believed to function as descending modulation of pain. Therefore, it may be implied that PIC participates in modulation of this descending pathway.

There were also labeled terminals in limbic areas, including medial thalamus (MD, CL and CM), dorsal peduncular nucleus and amygdalopiriform transition area. There is

no previous evidences revealing the connection between PIC and this areas, except the amygdala. Anatomical and behavioral studies both showed the projection of PoT-PIC pathway to amygdala, suggesting the contribution to relay the unconditioned stimulation (Shi and Davis, 1999). The labeling cells and terminals in basal ganglion, including striatum, globus pallidus, subthalamic nucleus and substantia nigra, may also due to the leakage to striatum. These data suggest that PIC may have function as pain processing and modulation.

In human functional image, PIC is believed to engage in pain processing. Edema or stroke of PIC could cause abnormal painful experience in clinical research, and lesion of PIC could induce a loss of nociception and negative emotion (Berthier et al., 1988; Isnard et al., 2011). Pain evoked by direct stimulation of PIC has also been reported (Mazzola et al., 2009; Ostrowsky et al., 2002). These evidences indicated that PIC modulates nociception in normal conditions. Neuronal tracing study revealed that strong PoT-PIC connection may be related to thalamocortical pathway about pain processing. Taking together, it can be suggested that the PoT-PIC pathway serves as a essential relaying pathway of nociception in pathological conditions.

In summary, PIC plays an important role in the maintenance of mechanical allodynia and, partially, the processing of cold allodynia. It is implied that mechanical allodynia, cold allodynia and spontaneous pain may be processed in differential

pathways. The PoT-PIC pathway may be the thalamocortical pathway which processing the evoked pain in neuropathic rats.

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Tables and Figures

Table 1. Abbreviations

AIC	anterior insular cortex	LPAG	lateral periaqueductal gray
AIP	agranualr insular cortex, posterior part	M1	primary motor cortex
APir	amygdalopiriform transition area	MD	mediodorsal thalamic nucleus
APT	anterior pertectal area	MGN	medial geniculate nucleus
BLA	basaolateral nucleus of anygdala	ml	medial lemniscus
CeA	central nucleus of anygdala	PIC	posterior insular cortex
CL	centrolateral thalamic nucleus	PIL	posterior intralaminar thalamic nucleus
CM	central medial thalamic nucleus	Po	posterior thalamic nucleus
cp	cerebral peduncle	PoT	posterior thalamic nucleus, triangular part
CPu	caudate putamen (striatum)	PRh	perirhinal cortex
DEn	dorsal endopiriform nucleus	S2	secondary somatosensory cortex
DLEnt	dorsolateral entorhinal cortex	SN	substantia nigra
DI	dysgranular insular cortex	STh	subthalamic nucleus
DP	dorsal peduncualr nucleus	VM	ventromedil thalamic nucleus
DRD	dorsal raphe nucleus, dorsal part	VP	ventral posterior thalamic nucleus
fr	fasciculus retroflexus	-VPL	-lateral part
GI	granular insular cortex	-VPM	-medial part
GP	globus pallidus	-VPpc	-parvicellular part
ic	internal capsule		

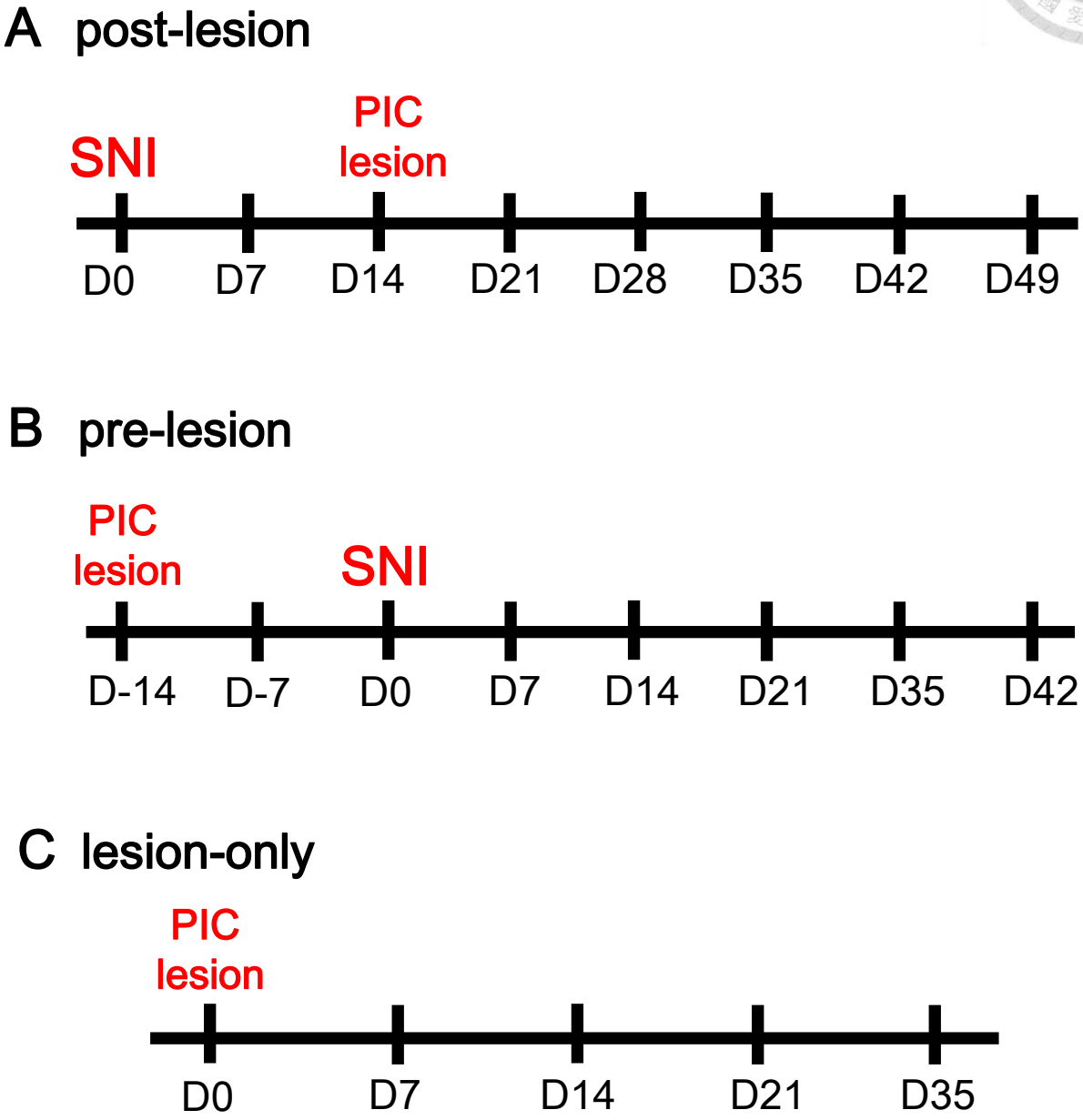

Figure 1. Diagrams of experimental procedures. (A) In post-lesion group, PIC lesion was performed 14 days after SNI. (B) In pre-lesion group, lesion was performed 14 days before SNI. (C) In only-lesion group, only PIC lesion was performed. Behaviors were tested at the specified dates.

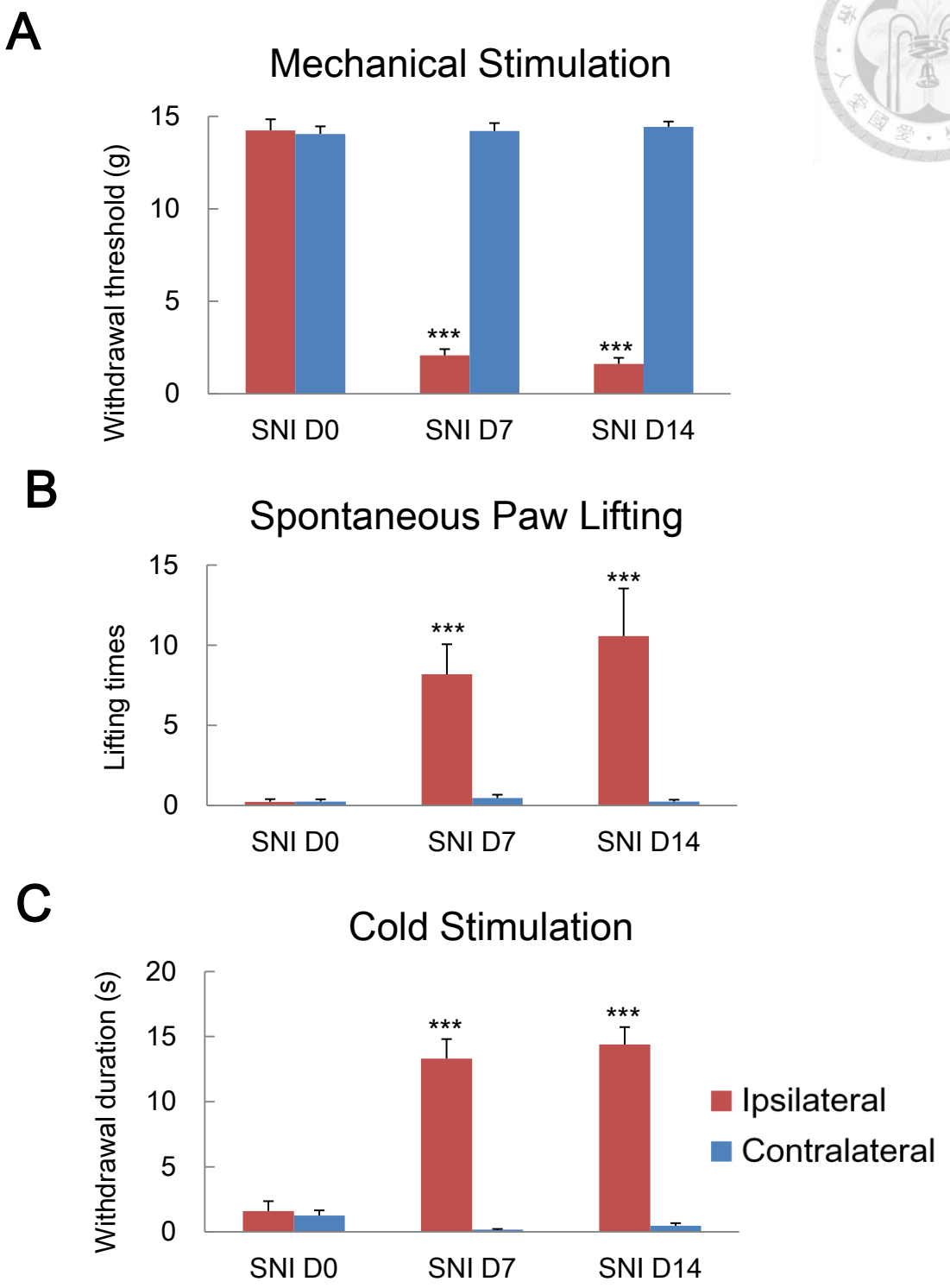

Figure 2. Behavioral changes before SNI, Day 7 and Day 14 after. (A) After SNI, rat developed mechanical allodynia on ipsilateral site. (B) Rats show spontaneous paw lifting on ipsilateral site after SNI. (C) Rats show increased paw lifting responding to acetone. *** $p \leqq 0.001$

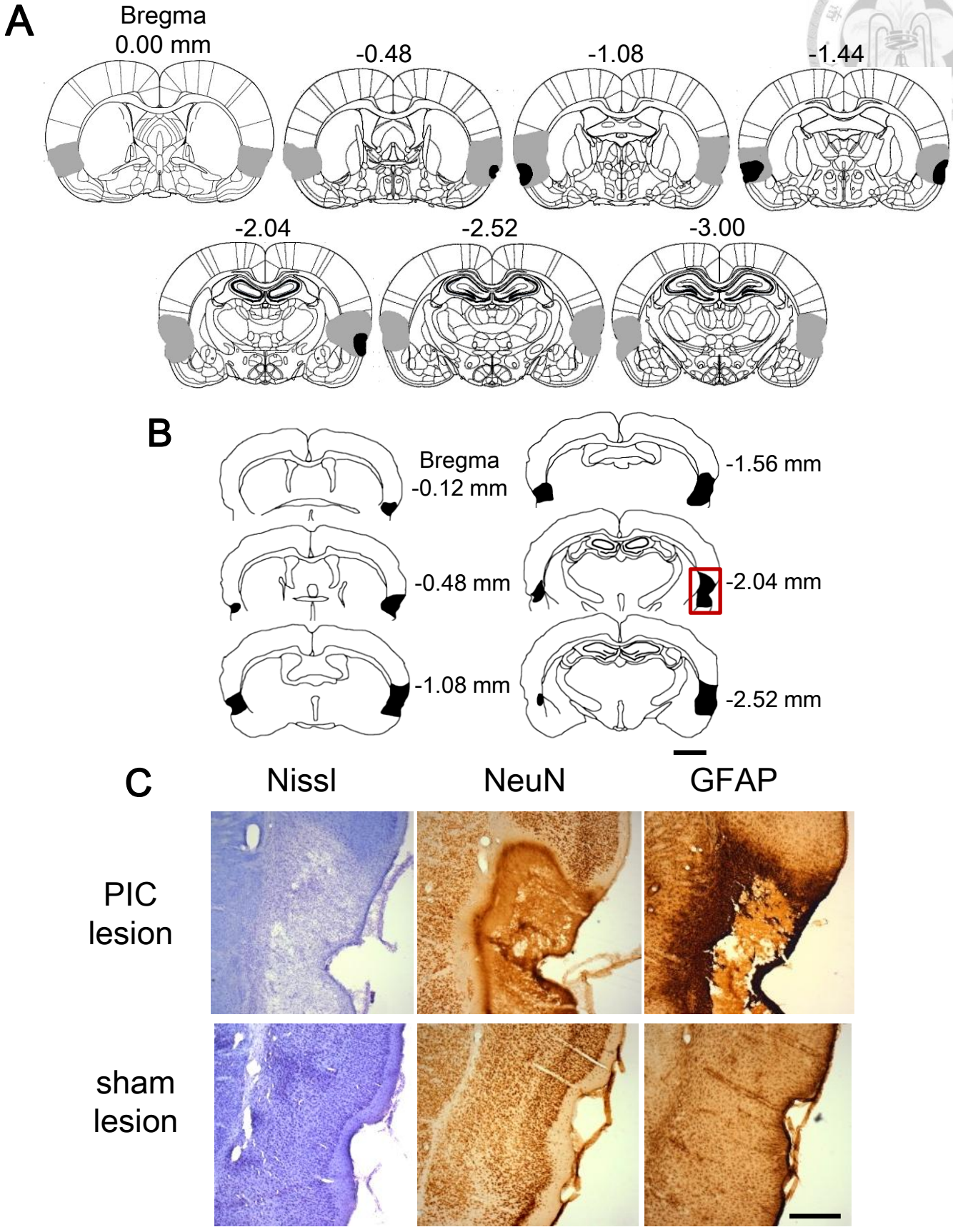

Figure 3. Extent of PIC lesion in the post-lesion group. (A) Summary of the smallest (black) and the largest (gray) lesion extent in PIC. (B) An example of the lesion extent in one rat. (C) Photographs of PIC lesion in red rectangle of (B) and the corresponding photos of a sham lesion rat. NMDA injected in the PIC caused obvious neuronal loss, tissue necrosis and gliosis.
Scale bar: 1 mm.

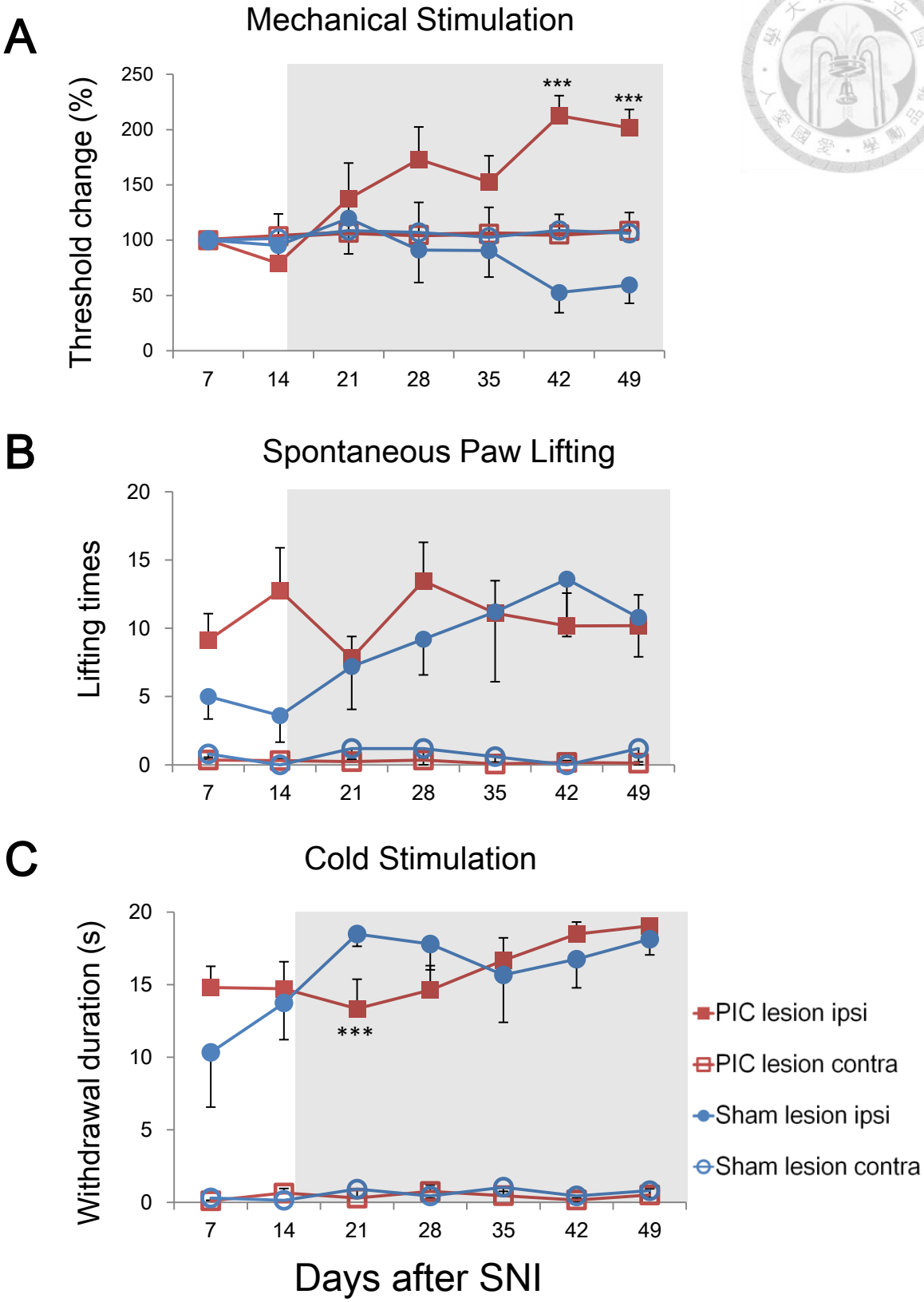

Figure 4. Behavioral changes of the post-lesion group. Bilateral PIC lesion was made at D14 after SNI (shaded area). (A) The withdrawal threshold to mechanical stimulation gradually recovered to two fold after PIC. (B) Spontaneous paw lifting shows no change before and after PIC lesion. (C) Withdrawal duration responding to acetone stimulation decreased on D21 but increased again on D28. ipsi: ipsilateral; contra: contralateral. *** $p \leqq 0.001$ compared to sham lesion ipsi.

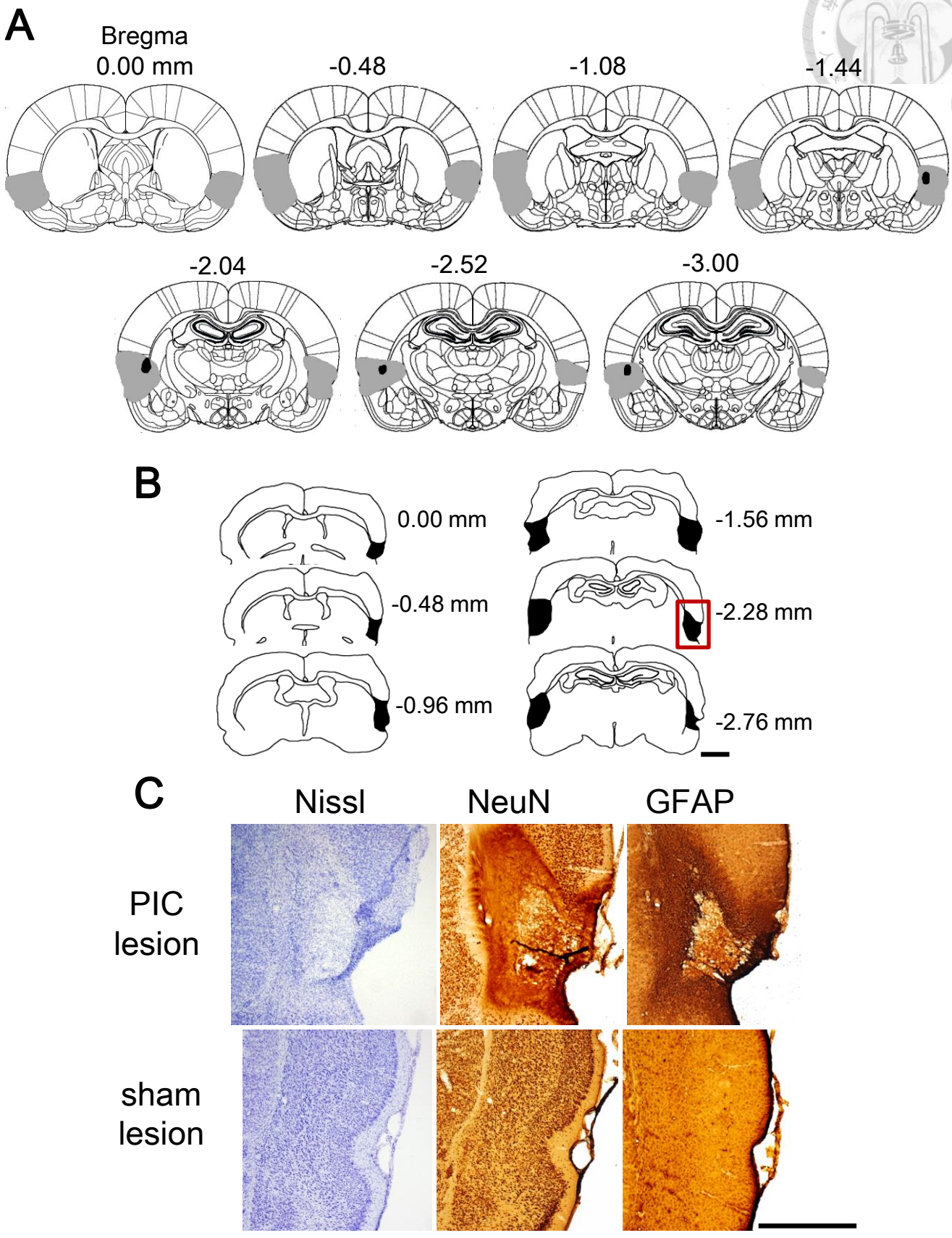

Figure 5. Extent of PIC lesion in the pre-lesion group. (A) Summary of the smallest (black) and the largest (gray) lesion extent in PIC. (B) An example of the lesion extent in one rat. (C) Photographs of PIC lesion in red rectangle of (B) and the corresponding photos of a sham lesion rat. NMDA injected in the PIC caused obvious neuronal loss, tissue necrosis and gliosis. Scale bar: 1 mm.

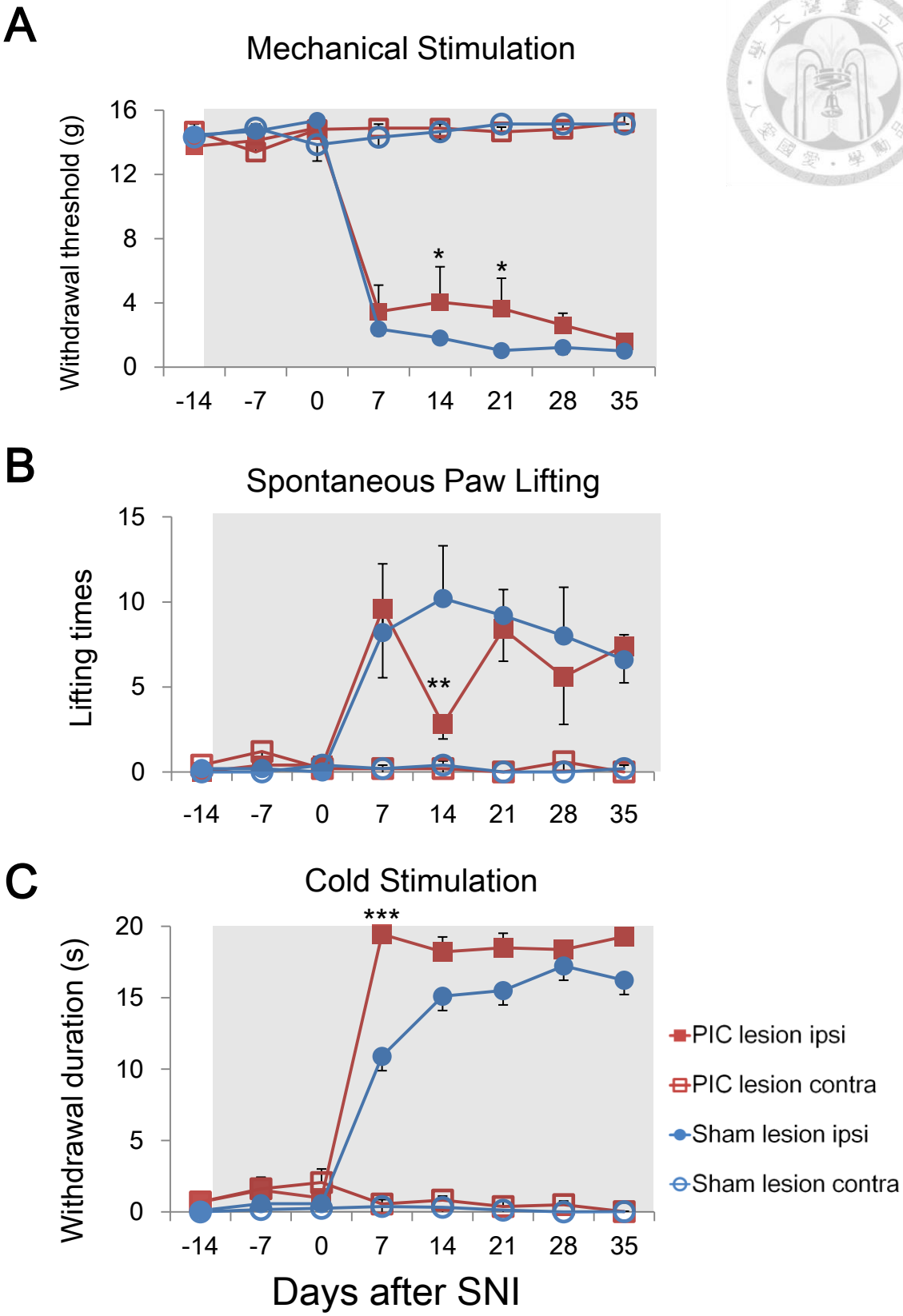

Figure 6. Behavioral changes of the pre-lesion group. Bilateral PIC lesion was made at D7 before SNI (shaded area). PIC lesion partially alleviated mechanical allodynia (A) and spontaneous paw lifting (B). However, PIC lesion caused higher withdrawal duration and faster development of cold allodynia (C). ipsi: ipsilateral; contra: contralateral. * $p \leqq 0.05$; ** $p \leqq 0.01$; *** $p \leqq 0.001$ compared to sham lesion ipsi.

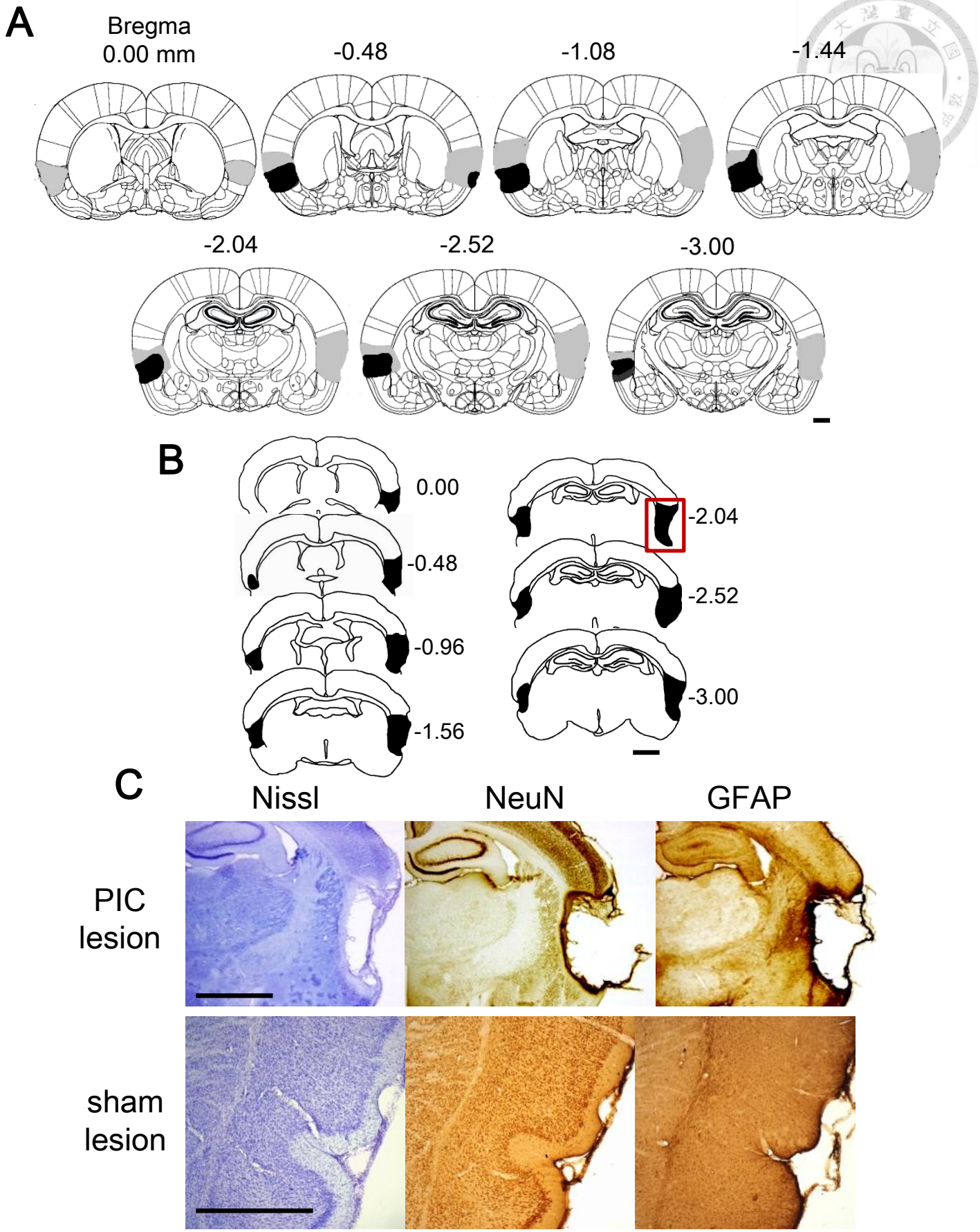

Figure 7. Extent of PIC lesion in the lesion-only group. (A) Summary of the smallest (black) and the largest (gray) lesion extent in PIC. (B) An example of the lesion extent in a rat. (C) Photographs of PIC lesion in red rectangle of (B) and the corresponding photos of a sham lesion rat. NMDA injected in the PIC caused obvious neuronal loss, tissue necrosis and gliosis. Scale bar: 1 mm.

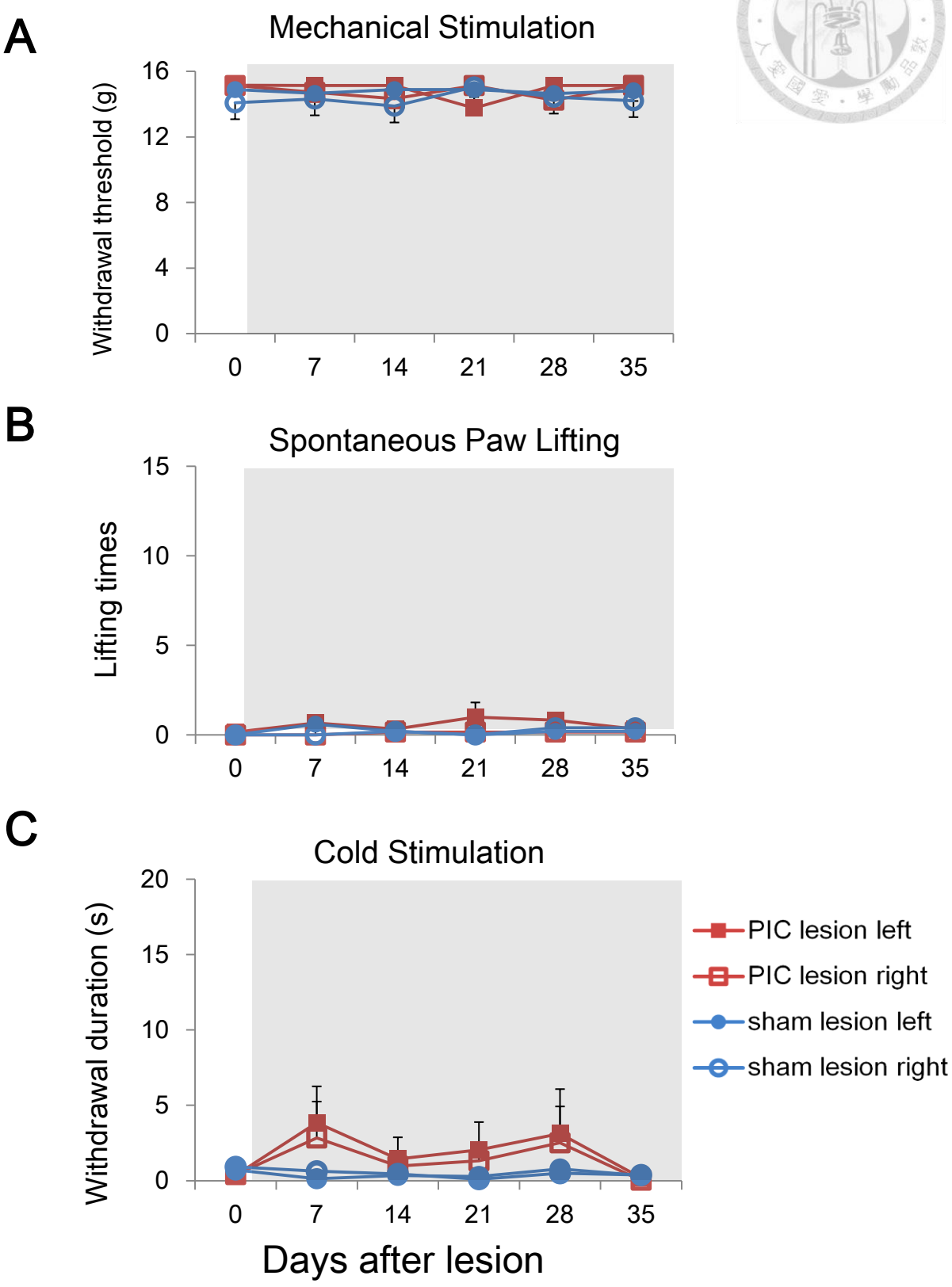

Figure 8. PIC lesion itself cused no significant change in withdrawal threshold to von Frey hair (A), spontaneous paw lifting (B), and withdrawal duration responding to acetone (C).

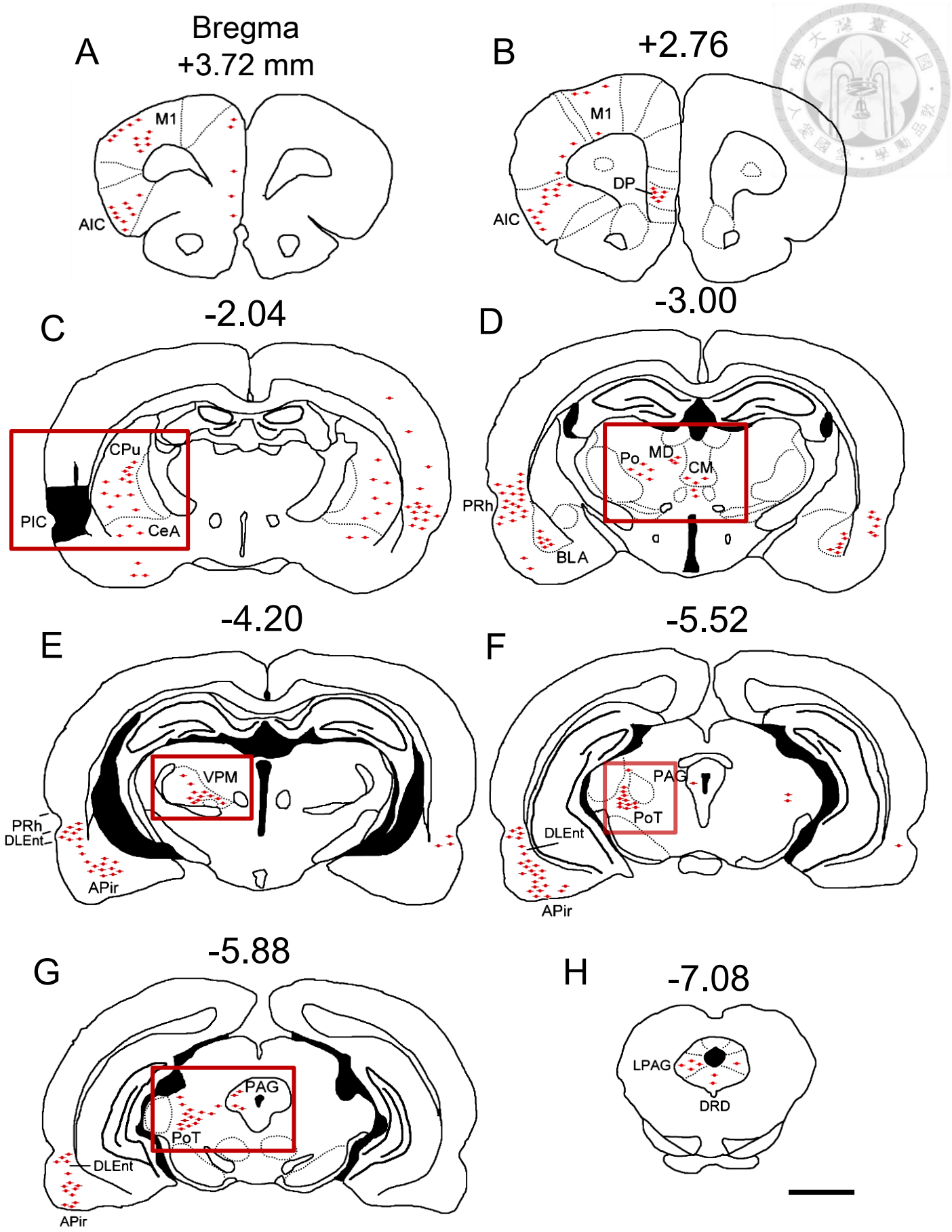

Figure 9. Series of drawings of labeled terminals through the brain in anterograde tracer study. Red dot symbols the terminal buttons. The magnifications of rectangles of C, D, F and G are present in Figure 10.
Scale bar: 1 mm

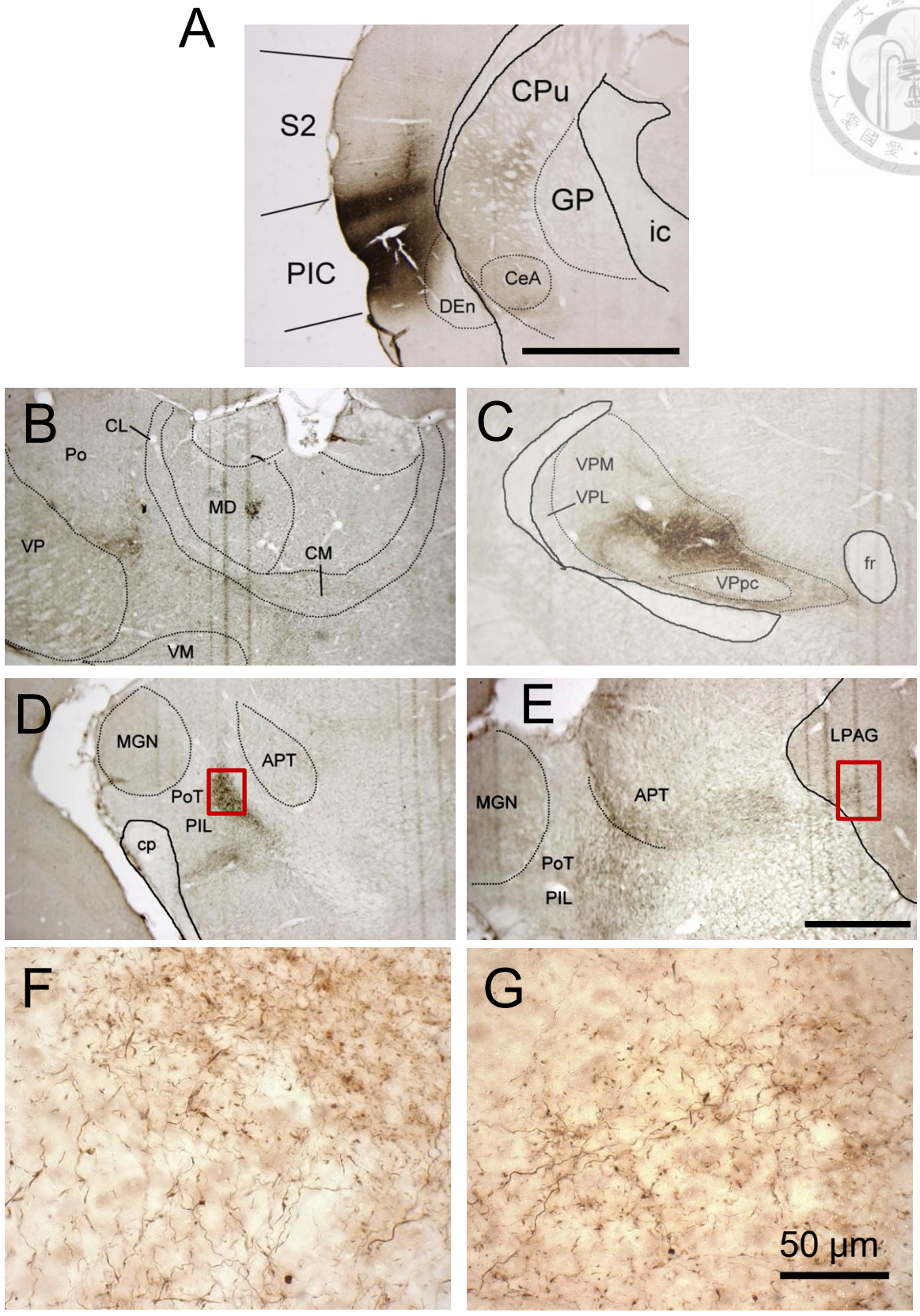

Figure 10. Magnification of the rectangles in Figure 9. Figure 10A corresponds to Figure 9C, 10B corresponds to 9D, 10C refers to 9E, 10D refers to 9F, and 10E corresponds to 9G. Figure 10F and 10G is the magnification of the rectangles in 10D and 10E, respectively.
Unlabeled scale bar: 1 mm.

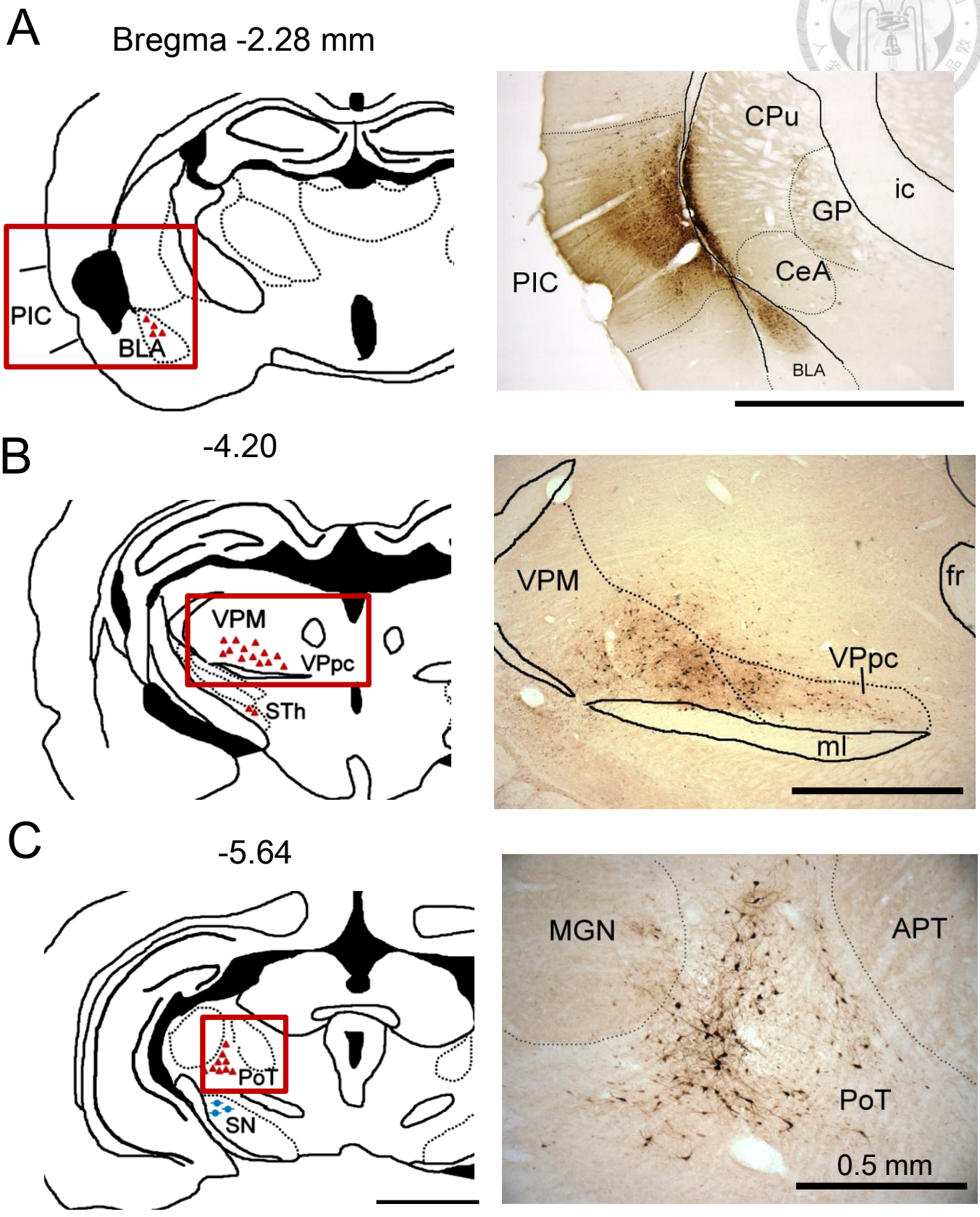

Figure 11. Series of drawings and labeled cell bodies of through the brain, and the corresponding magnification of rectangles in retrograde tracer study. Injection site is shown at Figure 11A. Cells and terminals are shown by red and blue symbols, respectively. Unlabeled scale bar: 1 mm.

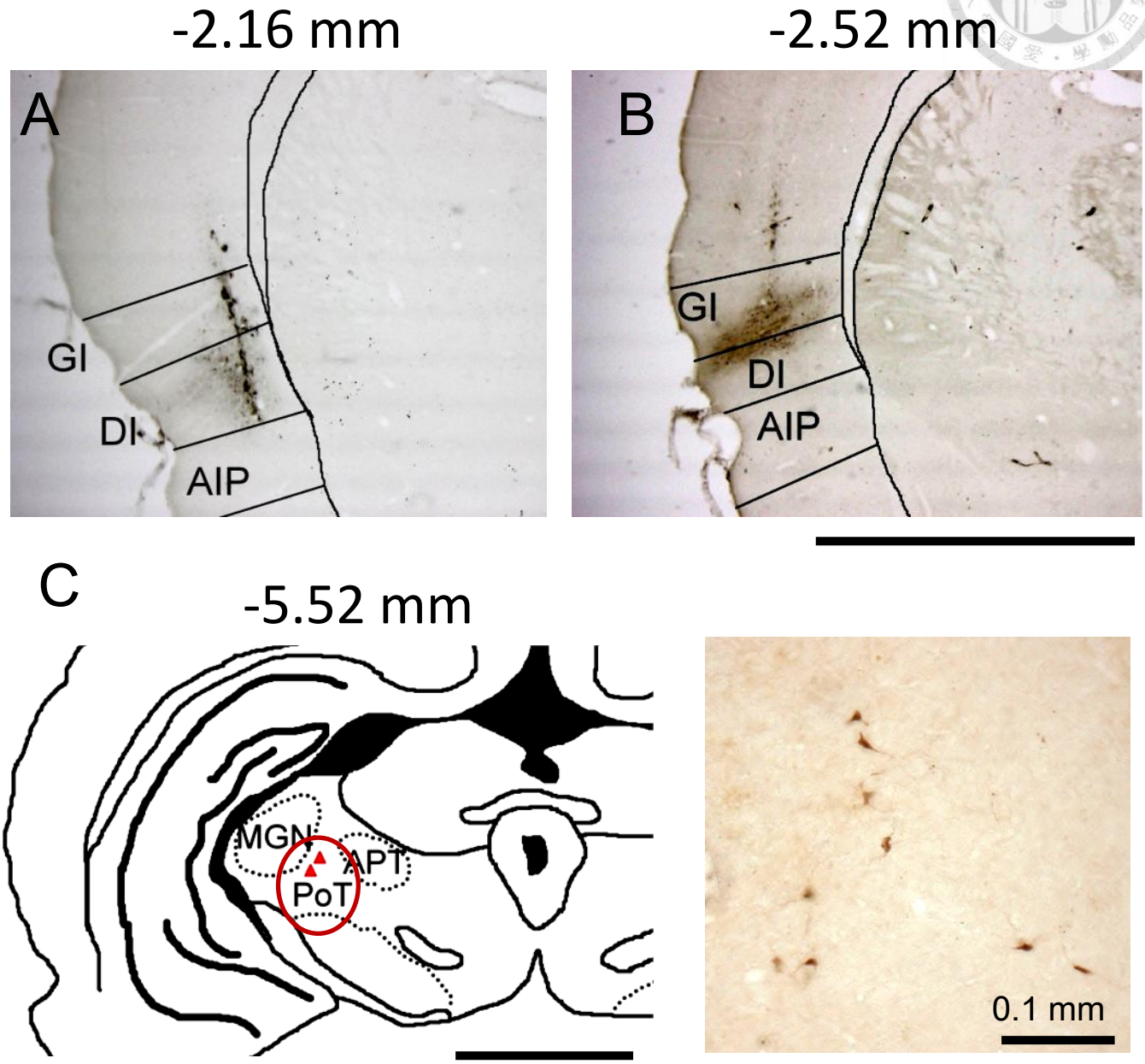

Figure 12. Injection sites of anterograde (A) and retrograde (B) tracer in small extent of injection. The labeled cell bodies are also drawn (C). Photograph of right panel is the magnification of red circle in left panel. Unlabeled scale bar: 1 mm.