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碩士論文

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小鼠背根神經節於神經痛之分子表現：以坐骨神經損傷為模式

Molecular Expression in Dorsal Root Ganglia of Neuropathic Pain Mice Induced by Spared Nerve Injury

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中文摘要

神經痛為周邊及中樞神經系統因疾病或原發性損傷所造成，患者感受到程度不一的異常感覺，如觸摸痛、痛覺過敏、痛覺減退等。由於症狀在臨床的表現複雜且機制尚未全面了解，藥物治療效果不佳且有耐受性問題，對於疼痛機制的研究以及開發有潛力的藥物治療標的有其必要性。周邊神經損傷為造成神經痛的其中一種原因，而三磷酸腺苷 (ATP)及嘌呤受體 (Purinergic receptor)的活化則為痛覺活化的可能途徑。其中 P2X3 受體於先前研究發現主要表現在中小型感覺神經元，因此我們使用小鼠坐骨神經損傷(Spared nerve injury, SNI)為模型，藉此探討 P2X3 受體及相關分子在背跟神經節(Dorsal root ganglion, DRG)之表現。

手術後小鼠手術肢機械性刺激閾值 (mechanical threshold) 下降而敏感化 (mechanical allodynia)，相對於非手術肢及手術前測試結果都有顯著差異。背跟神經節 L4 免疫螢光染色結果顯示，P2X3 受體主要表現於中小型神經元、以及少部分大型神經元; 而手術側與對照側之 P2X3 受體於整個神經元族群所佔比例並無顯著差異。利用雙重染色觀察 P2X3 型態上的分佈，主要和表現 lectin IB4 的 non-peptidergic 受體高度重疊，並且極少與表現 calcitonin gene- related peptide (CGRP)的 peptidergic 受體重疊，而 IB4/P2X3 神經元比例在手術側較對照側為低並達到統計上差異，因此表現 P2X3 的神經元族群於手術側與對照側可能有型態分佈上的改變。使用網路資料庫篩選參與 P2X3 啟動子結合調控的可能基因，其中 activating transcription factor-3 (ATF3) 之染色結果於手術側顯著上升，然而 ATF3 剔除小鼠接受 SNI 後表現之神經痛行為測試結果與 wild-type 組並無顯著差異，ATF3 與 P2X3 是否具有調控的相關性仍有待探討。

在 SNI 小鼠模型中，假手術組於機械性刺激閾值的絕對值與差值的統計結果呈現不一致的情形，代表需要更進一步探討小鼠的痛覺行為。此外，不同行為測量儀器、神經痛模型之間的行為與分子表現差異、免疫螢光染色中大型神經元在 P2X3 活化所扮演之角色、SNI 小鼠模型中受損與未受損神經元之分子表現、以及 P2X3 神經元族群分佈型態上的特性仍有許多可以探究的問題。

關鍵字：坐骨神經損傷模型 (SNI); 神經痛; P2X3 受體; 激活轉錄因子-3 (ATF3); 背跟神經節 (DRG); 痛覺受體

ABSTRACT

Neuropathic pain refers to pains resulting from disease or damage of the peripheral or central nervous systems, and from dysfunction of the nervous system. Patients suffer from this chronic, intensively painful experience that is difficult to treat with conventional analgesic medications. Peripheral nerve injury is one of the etiologies causing neuropathic pain, and ATP seems to be strongly implicated in peripheral pain sensitization. The purinergic receptor, P2X3, a ligand-gated cation channel, is expressed in a subset of small-diameter, primary afferent neurons, and might be a potential therapeutic target to relieve pain. Previous studies showed controversial results in terms of P2X3 expression in several peripheral nerve injury models. Some claimed that a functional up-regulation of P2X3 in dorsal root ganglia, whereas some reported contradicted results. We hope to investigate the regulation of P2X3 gene expression.

Using spared nerve injury model (SNI) in mice, we found animals displayed mechanical allodynia in the spared sural territory one week after surgery and persisted at least two weeks. From the results of immunofluorescent staining to estimate the P2X3 (+) neuron ratio on lumbar (L4) dorsal root ganglia (DRG), P2X3 receptors were mainly expressed in small and medium sized neurons, while few percentages of large neurons were also discovered both in the contralateral and ipsilateral sides. The P2X3 immunoreactive neuron ratios between ipsilateral and contralateral DRG did not show significant difference, and the distribution were highly colocalized with the nonpeptidergic neurons that express IB4, but rarely co-expressed with CGRP immunoreactive neurons, which are peptidergic. The (IB4/P2X3) neuron ratios in ipsilateral DRG were statistically lower than the contralateral DRG, suggesting a morphological shift of P2X3 distribution could be happened. To screen the possible

gene candidates regulating P2X3 expression on transcriptional level, we used bioimformatic web tools and further filtered by their functions. One possible molecule, activating transcription factor-3 (ATF3), was increased on the ipsilateral DRG significantly. Although the mechanical thresholds of ATF3-knockout mice after SNI were similar to those of the wild-type group, it is worth pursuing whether there exist relationship, probably with different neuropathic pain models.

In our study of SNI mouse model with mechanical allodynia, inconsistency of the sham group in difference of mechanical thresholds compared to absolute values, comparison of sensory abnormalities between dynamic plantar aesthesiometer and manual von Frey filaments, pain behaviors in different neuropathic pain models and species are possible issues to be investigated. From the results of immunofluorescent staining, quantities of large sized neuron in P2X3 (+) populations, degrees and location of nerve injury, and distribution of injured and intact neurons in DRG after SNI are also required to be further clarified.

Key words: Spared nerve injury (SNI); Neuropathic pain; P2X3; Activating transcription factor-3 (ATF3); Dorsal root ganglion (DRG); Nociceptor

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Chapter 1 Introduction

1.1 Neuropathic pain and peripheral nerve injury

Pain sensation is an essential signal for the survival of living creatures and human beings. It warns us from the potential dangers and prevents us from further injuries. Normal pain sensation results from the activation of nociceptors due to tissue damages. As injured tissue heals, the sensitization induced by peripheral and central mechanisms typically declines and the threshold for pain returns to pre-injury levels. However, when afferent fibers or central pathways themselves are damaged these processes can persist, and the resulting condition is referred to as neuropathic pain. The International Association for the Study of Pain (IASP) defines neuropathic pain as "pains resulting from disease or damage of the peripheral or central nervous systems, and from dysfunction of the nervous system". The prevalence of neuropathic pain is around 5- 8 % in the general population (Daousi et al., 2004; Stanislava Jergova, 2012) and it's difficult to treat with conventional analgesic medications.

Patients suffering from neuropathic pain undergo several sensory abnormalities; including (1) allodynia: pain due to a stimulus that does not normally provoke pain, (2) hyperalgesia: increased pain from a stimulus that normally provokes pain, and (3) hypoalgesia: diminished pain in response to a normally painful stimulus. Pain can arise spontaneously or be produced by mild stimuli that are common to everyday experience, like the gentle touch and pressure of clothing, or warm and cool temperatures. Reduced pain may also occur due to sensory denervation. The causes of neuropathic pain are clinically complicated, such as diabetic neuropathy, traumatic nerve injury, spinal cord injury, complications of HIV infection, multiple sclerosis, and stroke (John Scadding, 2003; Mark J. Lema, 2008)…. The diversity of the paradoxical symptoms makes it

difficult to classify and verify the mechanisms, and even the patients suffering from similar etiology do not necessarily develop identical condition of neuropathic pain. Current pharmacological medications are analgesics based on randomized clinical trials, includes antidepressants, opioid receptor agonists, or calcium channel blockers, in combination with other first-line therapies (Alec B. O'Connor et al., 2009; Ian Gilron et al., 2013; Alexandra Hovaguimian, 2011). With limited efficacy and problems of drug tolerance, researches on the mechanisms of neuropathic pain and development of therapeutic targets are extremely vital.

Peripheral nerve injury is one reason that causes development of neuropathic pain. At molecular level, how do the injured nerve and the intact uninjured nerve transduce the sensation of neuropathic pain? Various signaling molecules are produced and released followed by peripheral nerve injury, which stimulate appropriate receptors on sensory neurons, such as bradykinin, prostaglandin E2 (PGE2), serotonin, endothelin, nerve growth factor (NGF), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), ATP, proton (H^+), and glutamate, thus increase the excitability of primary sensory neurons through multiple signaling pathways (Ru-Rong Ji et al., 2004). ATP is a further strong candidate involved in peripheral pain mediation. Studies show that ATP can be released from primary afferent nerves themselves, endothelial cells, epithelial cells, and lysed cells from damaged tissues (R. Alan North, 2003; Kerstin Wirkner et al., 2007). Thus we focused our study on the molecular expression of pain molecules- especially purinergic receptors- on the corresponding dorsal root ganglia (DRG) of mice after peripheral nerve injury.

1.2 Purinergic receptors and pain

Purinergic receptors are classified into P1- activated by adenosine- and P2 receptor family that are activated by ATP or ADP. P2 receptors can be divided into P2X (1-7) (ligand- gated ion channels) and P2Y (1-8) (G protein- coupled receptors) according to their structures and signal transduction mechanisms (Abbracchio, M.P. et al., 1994; Khakh, B.S., et al., 2001). All P2X receptors are found on sensory neurons, whereas P2X3 has the highest level of expression, both in the mRNA and protein level (Geoffrey Burnstock, 2013). In 1995, P2X3 was cloned and shown to be localized predominately in a subpopulation of small nociceptive sensory neurons of DRG (Chen et al., 1995; Lewis et al., 1995). The distribution of P2X3 made scientists proposed the purinergic hypothesis for the initiation of pain and devoted into related researches, suggest that ATP released from nerve terminal, vascular endothelial cells and tumor cells… reaches P2X3 receptors on nociceptive sensory nerves (Geoffrey Burnstock, 2013). P2X3 receptors of DRG have been implicated in neuropathic pain. In studies of P2X3 knockout mice, the DRG did not show any current elicited by αβmeATP, the agonist of ATP, and the mice showed modest reduction of paw- flinching behavior after intraplantar formalin injection (R. Alan North, 2003; Souslova et al., 2000; Cockayne et al., 2000). The usage of P2X3 antagonists (e.g. TNP-ATP, A-317491, antisense nucleotides) on animals also showed reduced pain behavior (R. Alan North et al., 2012; Zhenghua Xiang et al., 2008; Yu-Lin Hsieh et al., 2012). At sensory nerve terminals in the periphery, P2X3 together with P2X2/3 receptors have been identified as the principal purinergic receptors present (Geoffrey Burnstock, 2013), and it was also involved in acute pain, inflammatory pain, chronic neuropathic pain, visceral pain and margine pain signaling (Wirkner et al., 2007; Alessia Franceschini et al., 2014; Anthony

P. Ford et al., 2013), further emphasized the importance of P2X3 receptors and the possibilities of developing therapeutic target to relieve pain.

1.3 Controversial Results of P2X3 Expression in Peripheral Nerve Injury Models

The researches in terms of P2X3 expression after peripheral nerve injury showed controversial reports in the past years. In different injury models, some claimed that a functional up-regulation of P2X3 in DRG, whereas some reported contradicted results- P2X3 was unchanged or even declined. In chronic constriction injury (CCI) model of rat, increased P2X3 have been found in DRG and spinal cord (Novakovic SD et al., 1999), the small and medium P2X3 positive neurons was increased using immunohistochemistry. In chronic compressed DRG (CCD) model of rat, P2X3 expression on the surgical group is higher than the sham control group, both in the results of western blot and immunofluorescent staining (Xiang et al., 2008). Similar result has been reported in chemical neuropathic pain model using the capsaicin analog-resiniferatoxin (RTX)- induced neuropathy of mice. Increased P2X3(+) neuron density on lumbar DRG accompanied by mechanical allodynia and thermal hypoalgesia have been found from D7 to D84 of treatment (Yu-Lin Hsieh et al., 2012).

However, in the research using spinal nerve ligation (SNL) model of rat, a significant reduction of P2X3 immunoreactivity on the lumbar DRG was also observed, both in the western-blot and immunohistochemistry (Karren Kage et al., 2002). In the spared nerve injury model (SNI) of rat, the expression level and distribution of total P2X3 was not significant different between the control and surgical group with the development of mechanical allodynia (Yong Chen et al., 2005). Together with these paradoxical findings,

the role of P2X3 in the mechanisms of peripheral neuropathic pain, its expression in different nerve injury models and different subtypes of DRG neurons, and the regulation of P2X3 gene expression remain to be investigated.

In our study, we set up the spared nerve injury model of mice and evaluated the mechanical pain abnormalities using dynamic plantar aesthesiometer. First published in rat, spared nerve injury produced robust, reliable, long- lasting neuropathic pain- like behaviors (allodynia and hyperalgesia) that mimic many clinical features in human, and it provides the possibility of studying the injured and uninjured neurons in the same spinal ganglia (Marie Pertin et al., 2012, Decosterd et al., 2000). With the partial nerve injury of the mouse sciatic nerve, we hope to investigate the gene regulation of P2X3 receptors in pain state on transcriptional level, and to figure out the putative transcription factors that have interaction with P2X3 gene. Activating transcription factor-3 (ATF3), a member of the mammalian activation transcription factor/cAMP responsive element-binding (CREB) protein family of transcription factors, is widely used as a marker for injured DRG neurons in pain research (Hiroaki Tsujino et al., 2000; Hans Lindå et al., 2011). Screening from the bioimformatic websites, we found ATF3 a possible candidate that could influence the P2X3 gene expression, so we have also used ATF3-knockout mice manipulated with SNI to observe their pain behaviors and to investigate the relationship between ATF3 and P2X3 protein expression through immunofluorescence. The ATF3-knockout mice lack functional ATF3 genes, and they have no lethality or obvious phenotypes, consistent with the notion that ATF3 is a stress-inducible gene and is not required under normal conditions (Matthew G. Hartman et al., 2004).

To resolve the distribution of P2X3 in representative DRG subgroups, we have conducted the double staining with calcitonin gene-related related peptide (CGRP), a

marker of peptidergic nociceptors, Isolectin IB4, a marker of nonpeptidergic nociceptors, and SMI-32 (non-phosphorylated neurofilament H 200kDa) as a marker for large-sized neurons. During sensory neurogenesis of the perinatal and postnatal development stage, the nociceptors differentiate into two major classes: the nonpeptidergic and peptidergic neurons with distinct repitores of ion channels and receptors. The nonpeptidergic nociceptors switch off TrkA receptor to NGF and begin to express Ret, the component of the receptor to glial cell- derived growth factor (GDNF), accompanied with the expression of Runx1 transcription factor, which are IB4 (+). The rest retain TrkA expression and develop into peptidergic nociceptors that express CGRP and substance P (SP) and are IB4 (-) (Clifford J. Woolf et al., 2007; Gregory Wynn et al., 2004). We hope to investigate the changes of molecular expression in the DRG of mice after SNI on the post-translational level using immunofluorescence.

Chapter 2 Materials & Methods

2.1 Animals

Male adult C57BL/6J mice were purchased from National Taiwan University College of Medicine Animal Center as the wild-type group to undergo the SNI surgery, and the strain of ATF3-Knockout mice was a general gift from Prof. Hai (Matthew G. Hartman et al., 2004). Maintained in the Animal Center, the inbred strain of the KO mice were mated by homozygous individuals. All animal procedures were approved by the animal committee of National Taiwan University College of Medicine and were conducted according to Guide for the Care and Use of Laboratory Animals from National Research Council.

2.2 Spared nerve injury model

We set up the spared nerve injury model (Decosterd and Woolf, 2000). It involves the ligation and transcetion of two of the three branches of the sciatic nerve (the common peroneal nerve and tibial nerve) while the thinnest sural nerve was left intact. Animals were anesthetized by sioflurane inhalation. After shaving the hair and incising the skin on the lateral surface of the right thigh, the biceps femoris muscle was stretched, exposing the sciatic nerve and the accompanied trifurcation on the distal terminal. The common peroneal nerve and tibial nerve were ligated with 6.0 surgical sutures and transected for 1mm distal to the ligation to avoid nerve regeneration. It was critical not to touch or damage the sural nerve on the posterolateral side of the trifurcation. The superficial cutaneous branch and blood vessels should also be carefully preserved, otherwise, the experimental results could be strongly biased (Mette Richner et al., 2011; Marie Pertin et al., 2012). The muscle layers were gently covered and the wound was

sutured to avoid improper inflammation. In the sham surgery group, mice were manipulated by similar surgical procedures compared to the SNI group without damaging the sciatic nerve.

2.3 Animal behavior evaluation

Sensory function evaluation of the mice was conducted with the mechanical test using Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy). The rigid metal tip of the aesthesiometer, with a diameter of 0.5mm, was applied to the posterolateral region of the hind paw between the 2nd and 3rd pad on both limbs, which was the sensory territory innervated by the spared sural nerve. When the mice received the noxious stimuli of increasing force (0 to 15g in 30s) to some extent, they typically displayed the pain behavior- paw withdrawal, flinching, and paw licking, and the mechanical thresholds (g) were recorded by the device. The mechanical tests were conducted blinded before the surgery, Day 3, and Day 7 post-surgery. The mice were acclimated in the testing room environment and devices 1 to 2 day before the pretest to reduce the anxiety, and for each day of the test, the mice were habituated 1 to 2 hr in the diurnal hours prior to the test. Laboratory coats, gloves and masks are equipped to minimize odor disturbance. Stimulation on the animal with quiet and motionless posture could be regarded as a valid data.

2.4 Immunofluorescent staining of DRG

The animals were sacrificed 7-9 days after SNI surgery by intraperitoneal injection of 4% chloral hydrate (0.01 c.c./g) and the DRG tissues were fixed by transcardial perfusion of 1% sodium nitrite and 2% paraformaldehyde followed by post-fixation for

2 hr. The composition of mouse sciatic nerve- L3 to L5 DRGs (Marcel Rigaud et al., 2008; Cédric J Laedermann et al., 2014) were dissected and stored in 0.1M phosphate buffer (pH 7.2) in 4 ℃. The DRGs were then cryoprotected with 30% sucrose for overnight and embedded by cryochrome in -20 ℃ for cryostat sectioning (LEICA CM3050-S). For each slide, 6-8 sections (8 μm in thickness) with an interval of 70 μm were immunostained followed by the general procedure established in our lab. The sections were washed by 0.5M Tris buffer three times, blocked in 0.1% non-fat milk with 0.5% Triton X-100 in Tris for 1 hr to avoid non-specific binding, and incubated in the primary antisera for overnight in 4℃. The primary antisera include P2X3 (rabbit, 1:2000, Neuromics, Edina, MN), ATF3 (rabbit, 1:100, Sigma), Neurofilament SMI32 (mouse, 1:2000, Covance, Emeryville, CA), CGRP (goat, 1:500, AbD SeroTec), and biotinylated IB4 lectin (1:100, Sigma). In the second day, the sections were washed and incubated with Alexa Fluor 488-, Cy3-, or Alexa 647- conjugated secondary antisera (1:100, Jackson ImmunoResearch) corresponding to the hosts of primary antisera for 1h and accompanied mounting. The secondary antiserum of IB4 was Cy3- conjugated streptoavidin. The contralateral DRGs were used as negative control. The scale bars on the following figures are 100μm in length.

2.5 Genotyping of the mice

To identify the genotype of each mouse, 0.5 cm of the fresh tail was cut for genomic DNA extraction (Promega DNA purification kit, A1125) and the sequence of manipulated allele was amplified by PCR using three primers (Matthew G. Hartman et al., 2004). The molecular weight of the PCR product was identified by 2% agarose gel electrophoresis, with 100ng per well. The wild-type allele was 329bp, and the knockout

allele was 236bp.

2.6 Bioinformatic web tools

In order to study the regulation of P2X3 gene expression on transcriptional level, the putative promoter sequence of rat P2X3 gene was accessed by the Transcriptional Regulatory Element Database, and the Prokaryotic Promoter Prediction was used to predict the possible protein structures that might bind to the sequence. The possible candidate genes encode functional products that have interaction with the promoter were surveyed by Ensembl, NCBI Gene, and Homologene websites. Further investigation of the candidate genes were filtered by the gene function (e.g. sequence-specific DNA binging transcription factor activity, molecules involved in neuron development, nerve injury and pain). The remaining gene candidates were listed in the **Table 1**, with the full name, abbreviation of the genes, and the general description. The species of the listed genes are *Rattus norvegicus* (Norway rat).

2.7 Data analysis

In statistical analysis, all data are expressed as mean±SD. Animals with discrete values of behavioral data were excluded in the following analysis, i.e., differences of mechanical threshold on pretest that beyond mean±2SD. In mechanical behavioral tests, the mechanical thresholds (g) between contralateral and ipsilateral sides and the difference of mechanical thresholds (g) compared to pretest were analyzed by paired-t test. The P value < 0.05 was considered to be statistically significant. Comparison between two genotypes of mice was analyzed by unpaired-t test. The linear regression of the mechanical thresholds conducted by two operators was computed by

nonparametric Spearman correlation. For the quantitation of immunoreactive neurons in DRG, each section was randomly chosen from each slide at 10x object under the fluorescence microscope (Zeiss AxioImager, A1) and photographed under identical exposure time (Camera: Monocolor camera: ZEISS AxioCam MRm1388*1040P) (Software: Axiovision). Quantitation of the photos was conducted blinded, only the neurons with complete circular profile were counted, and the intensities of the neuron in gray scale were measured by Image J 1.47v. The total neuron number for each section was counted by the DIC microscopy under bright-fields. The ratios of the positive neurons between contralateral and ipsilateral DRGs were estimated by paired-t test.

Chapter 3 Results

3.1 Neuropathic pain behavior- mechanical allodynia induced by SNI

To explore the phenotypic changes in neuropathic pain behaviors, we first conducted the mechanical test in a series of rigorous procedures using the automatic dynamic plantar aesthesiometer. The mice with SNI exhibited mechanical allodynia with changes in mechanical thresholds on D3 post-surgery, and changes became stronger to D7 **(Figure 1)**. Reduced mechanical thresholds in the ispilateral side compared to the contralateral side showed significant difference on D3 and D7 ($p<0.0001$), whereas the hypersensitivity was not observed in the mice subjected to the sham surgeries. In the difference of mechanical thresholds (g), i.e., mechanical threshold on the ipsilateral side minus the contralateral side **(Figure 2A)**, theoretically for each mouse the difference on pretest will close to 0 g. The data displayed negative values on D3 and D7, with the mean close to 2 g (D3= -1.92g; D7= -2.3g), and also had significant difference in comparison with pretest ($p<0.0001$). Although we have some inconsistent results on D3 of the sham group **(Figure 2B)**, the p value was< 0.05 on D3 compared to pretest, it is possibly considered to be the issues of sample size and the variation on the day of pretest.

In our model, this mechanical allodynia- like hypersensitivity persists at least 2 weeks (D14) post-surgery **(Figure 3A)**, and previous researches suggested this phenomenon could persist at least 4 weeks to 6 months (Anne-Fre'de'rique Bourquin et al., 2006; Amanda K Smith et al., 2013). The difference of mechanical thresholds from D3 to D14 showed significant difference in contrast with the pretest thresholds ($p<0.05$), and the absolute mechanical thresholds on the ipsilateral limbs from D3 to D14 also showed

significant difference from pretest and without significant difference within these days. Also, we have conducted the mechanical tests twice by two different operators with the same individual mice and on the same day. The results displayed positive correlation between two operators (r^2= 0.3655, p<0.0001) **(Figure 3B)**. The experimental data revealed minimal difference of the technical procedure in distinct person, which means the operators showed identical criteria to the withdraw behavior and animal handling. Together with the condition of blind tests, the subjective bias and psychological expectations of the operator could be excluded, so the mechanical allodynia of the mice with SNI was highly reliable.

3.2 P2X3 expression in the lumbar DRGs after SNI

To examine the molecular changes in corresponding lumbar DRGs after SNI, the animals were sacrificed 7-9 days after the operation, and the immunofluorescent staining recognized by specific antibodies were used. First we have identified the distribution of injured neurons in different levels of the lumbar DRG after SNI. The spinal ganglia of mouse sciatic nerve were composed of L3, L4, and to a lesser extent, L5 DRG (Marcel Rigaud et al., 2008; Cédric J Laedermann et al., 2014), so we have used activating transcription factor-3 (ATF3) as a nerve injury marker to calculate the cell bodies of injured common peroneal and tibial nerve from L3 to L5 DRG **(Fig. 4A)**. In L3 and L4, about 40% of neurons are ATF3 (+), and 2.7% of neurons in L5 are ATF3 (+) **(Fig. 4B)**. This suggested that most of the injured peroneal and tibial neurons are located in L3 and L4, whereas few were found in L5 DRG. In a study using SNI-variant with spared common peroneal and tibial nerve, the ATF3 immunoreactive cells was increased in L4 (17%) and L5 (15%), suggesting that the spared uninjured sural nerve

mostly projects into L4 and L5 DRG (Cédric J Laedermann et al., 2014). From the above results we concluded to compare the molecular expression on the most representative L4 DRG.

Using anti-P2X3 and SMI32 antibodies, The P2X3 receptors were mainly expressed in small and medium sized neurons, while few percentages of large neurons were also discovered both in the contralateral and ipsilateral DRG **(Fig. 5A)**. The P2X3 immunoreactive neurons on the ipsilateral side were not significantly different from the contralateral side in the L4 DRG of SNI mice (16±4.9 % v.s 16.1±4.9 %, p= 0.9796) **(Fig. 5B)**. In the quantative data of P2X3 (+) neuron in SMI32 (+) population, about 5% of SMI32 (+) neurons were colocalized with P2X3 **(Fig. 5C)**. The P2X3 (+) neurons in the large-diameter subgroup showed a mild increase in the ipsilateral side, but the p value does not show statistical meaning. Perhaps the large diameter Aβ fiber plays certain role in the SNI neuropathic pain sensitization.

To further verify the distribution of P2X3 receptor in the subpopulation of DRG, we have conducted the triple staining of P2X3 with IB4 and CGRP **(Fig. 6A)**. P2X3 receptors were highly colocalized with the nonpeptidergic neurons that express IB4, but rarely co-expressed with CGRP immunoreactive neurons, which were peptidergic. The double staining and quantitation of P2X3+CGRP, P2X3+IB4, and CGRP+IB4 showed that CGRP (+) neuron ratio and IB4 (+) neuron ratio between the contralateral and ipsilateral DRG have no significant difference (CGRP (+)= 16.3± 8.14% v.s 14.29± 2.11%, p= 0.6011; IB4 (+)= 32.54± 2.64% v.s 30.87± 3.98%, p= 0.3865), and only about 5% of P2X3 (+) neuron colocalized with CGRP (+) neuron (8.44± 6.6% v.s 6.04± 0.84%, p=0.5286), consistent with previous research data **(Fig. 6B, 6C)**. To take notes, the high ratio of P2X3 (+) neuron colocalized with IB4 was declined in the ipsilateral

DRG compared to the ratio in the original contralateral DRG (72.29± 6.16% v.s 87.47± 3.67%, p= 0.0048) **(Fig. 6C)**, suggesting that the morphological distribution of P2X3 receptors in L4 DRG could have changed after SNI treatment, since the unchanged P2X3 immunoreactive neuron ratio from the previous data. Scarce colocalization of peptidergic CGRP (+) neurons and nonpeptidergic IB4 (+) neurons was also illustrated, and only about 1% of total neurons were colocalized with CGRP + IB4 (data not shown) **(Fig. 6B)**.

3.3 Increased expression of ATF3 in the ipsilateral DRGs and the assessment of neuropathic pain behavior of ATF3-knockout mice receiving SNI

To screen possible gene candidates probably have interaction with P2X3 gene on transcriptional level, we found that the immunoreactivity of activating transcription factor-3 (ATF3)– one of the putative gene candidates– was dramatically increased on the nuclei of ipsilateral DRGs in mice after SNI **(Fig**.7A). It is widely used as a neuron injury marker in peripheral pain research, as previously discribed (Hiroaki Tsujino et al., 2000; Hans Lindå et al., 2011). The quantitative data **(Fig. 7B)** showed about 35% of the neurons were ATF3 (+) on the ipsilateral side, whereas few ATF3 (+) neurons were found on the contralateral side (p= 0.0002). This phenomenon was close to an all-or-none result.

On the other hand, we wanted to investigate the role of ATF3 in the sensory function of pain, so we observed the pain behavior of ATF3-knockout mice after SNI manipulation. In the mechanical test, decline of mechanical thresholds on the ipsilateral side compared to the contralateral side suggests that ATF3-knockout mice also

displayed mechanical allodynia ($p < 0.0001$) **(Fig.8)**. In the sham group of ATF3-knockout mice, no significant difference between the two sides was observed. The difference of mechanical thresholds on D3 and D7 also showed negative value (mean: D3= -1.76g; D7= -1.47g) with significant difference compared to the pretest ($p<0.05$) **(Fig.9A)**. In the sham group of ATF3-knockout mice, inconsistent result on D3 compared to pretest ($p < 0.05$) could be attributed to sample size and an outlier of unspecified individual **(Fig. 9B)**. We have also confirmed the genotypes of wild-type and ATF3-KO mice from the results of genotyping. The KO allele was slightly shorter than the wild-type allele (236 / 329 bp) **(Fig.10A)**.

To further analyze the mechanical allodynia between wild-type and ATF3-knockout mice after SNI, comparison of the difference of mechanical thresholds on each day between these two genotypes was displayed **(Fig.10B)**. The value of the ATF3-knockout mice seemed to be slightly less than the wild-type mice post-surgery, but they have no significant difference. This indicates without functional ATF3, the mice still displayed mechanical allodynia after SNI surgery. ATF3 was not involved in the neurotpathic pain sensation or played a minor role in the signal transduction induced by SNI. The importance of ATF3 still cannot be fully excluded, given the obvious up-regulation in injured neurons, and its role of stress-inducible gene.

Chapter 4 Discussion

In our research, we studied the molecular expression in the DRG of neuropathic pain mice with spared nerve injury using immunofluorescent staining. By the specificity of antigen- antibody recognition we can estimate the target protein expression and morphological distribution in fixative sections. The wild-type and ATF3-knockout mice displayed mechanical allodynia with the device of dynamic plantar aesthesiometer and persisted at least 2 weeks. This is consistent with previous studies of SNI established in mice using classical von Frey filament (Anne-Fre'de'rique Bourquin et al., 2006) that the phenomenon persisted for 4 weeks. While previous studies of P2X3 receptor regulation following nerve injuries showed variable results, our data implied the P2X3 protein expression on the representative L4 DRG of SNI mice was not significantly different between the ipsilateral and contralateral sides. This is consistent with the finding of a study using SNI rat model (Yong Chen et al., 2005) yet enhanced P2X3-mediated responses was observed. The characteristic distribution of P2X3 in DRG was highly colocalized with IB4 and rarely colocalized with CGRP in the origin, while morphological changes after SNI was observed that the IB4/P2X3 ratio declined slightly but significantly in the ipsilateral DRG. Dramatically increased ATF3 revealed nerve injury-induced neuronal activation on the lumbar levels of DRG corresponding to the mouse sciatic nerve, which was slightly different from the anatomical distribution in rat. There are still many questions required further validation, and several issues need to be investigated.

4.1 Mechanical allodynia of SNI mouse model using dynamic plantar aesthesiometer

SNI was the animal neuropathic pain model first published in rat with consistent neuropathic pain behaviors. With extended development to different strains of mice, variants of injury site and devices of sensory function evaluation, mechanical thresholds of the mice showed some inconsistent data in the past years. Reduced mechanical withdrawal thresholds were published in mice using von Frey filaments (Anne-Fre'de'rique Bourquin et al., 2006; Amanda K Smith et al., 2013). In the study of SNI variant- spared tibial nerve- in transgenic mice, mechanical allodynia using von Frey filaments together with thermal hyperalgesia were displayed (Dimitra Terzi et al., 2014). However in another study on different variants of SNI mice, only SNIt- spared tibial nerve- displayed mechanical allodynia with von Frey stimulation; while spared common peroneal or sural nerve the pain behavior of mice did not show significant difference compared to sham groups, and paw withdrawal latency to thermal stimulation was unchanged after any of the nerve injury models tested (Shannon D. Shields et al., 2003). In our study, we established the SNI mouse model with significantly reduced mechanical threshold according to the device of dynamic plantar aesthesiometer.

Reduced mechanical thresholds could be allodyia or hyperalgesia, clinically we can discriminate these sensory abnormalities by the responses of patients and their description, since pain is a sort of subjective perception. Pain estimation of animal models relies on the behavioral tests, and the devices function with different principles or parameters might bring to variable outcome. In classical manual von Frey filaments using up- down paradigm, a series of monofilaments was applied to the plantar region of the hindpaw once at a time, and changes of the caliber of monofilaments were

according to the animal responses. In dynamic plantar aesthesiometer, a stably increasing force was applied to the sensory territory of the hindpaw with a rigid metal tip. In previous studies, reduced mechanical threshold in different pain models conducted by von Frey filaments followed by the above principle are commonly called mechanical allodynia (Decosterd et al., 2000; Yong Chen et al., 2005; Yu-Lin Hsieh et al., 2012), while this phenomenon conducted by dynamic plantar aesthesiometer was not that consistent. Mechanical allodynia, mechanical hypersensitivity, and mechanical hyperalgesia were also published in different animal and neuropathic pain models conducted by dynamic plantar aesthesiometer, and the values of mechanical thresholds published in similar pain models varied, too(Umut İrfan Üçel et al., 2015; Paul J. Austin et al., 2012; Kata Bo¨lcskei et al., 2005; Yeon Jang et al., 2012). Comparison between these two devices in identical injury models implies similar tendency yet not totally the same in some injury models. Paw deformity due to foot postures in different injury types might be a potential issue that influences the mechanical threshold recording (Ramakrishna Nirogi et al., 2012). Here the reduced mechanical threshold in our SNI mice is referred to as mechanical allodynia, and the withdraw recordings of each individual using two different devices still requires further analysis.

Absolute mechanical threshold is the most common form to display, and the difference of mechanical threshold is also accessible. Here we presented the results of mechanical test in both formalities. The data showed some discrepancy in the sham groups, and different biological meanings to the pain behavior of the animals were disclosed. In the form of absolute values, comparison was conducted between two limbs on the same day and identical environment, and this is on the assumption that nerve injury didn’t induce any central effect to change the mechanical threshold on the control side, or the animal behavior was not altered by learning and memory constitution. In the

form of differences, comparison was conducted between specific day post-surgery and the pretest, which was the true original state of the animal sensation, presumably close to 0 (g). However, statistics of the difference of mechanical threshold could be strongly biased by an extreme value on the day of pretest, and factors that could increase the difference on two limbs should be minimized. Both formalities include advantages and possible problems that might be ignored. Possible factors were involved in the inconsistence of p value significance in the sham groups of difference of mechanical threshold. Sample size could be increased, and the differences were more complicated and variable than absolute values even though the extreme values were excluded, thus may affect the statistical results.

Our knowledge on the mechanisms of neuropathic pain is mainly based on animal models partially mimic some of the clinically observed symptoms in patients of neuropathic pain (Stanislava Jergova et al., 2012). Different degree and location of injuries may lead to variable sensory abnormalities. Most of the models are surgeries manipulated on the sciatic nerve or part of the spinal cord. Sciatic nerve axotomy is the transection of the sciatic nerve, which mimics the paradoxical symptoms of phantom limb, but the intense sensory denervation and loss of motor function make the pain behavior tests inaccessible. Partial ligation of the sciatic nerve and chronic constriction injury involve the ligation of nerve fibers and various degrees of injured neurons in the corresponding sensory ganglia. Animals displayed thermal and mechanical hyperalgesia (Paul J. Austin et al., 2012) that mimic the nerve trauma of causalgia. Further modified spinal nerve ligation model restricted the injured spinal ganglia within L5 DRG, and the adjacent L4 DRG could be studied as intact uninjured spinal ganglia (Stanislava Jergova et al., 2012; Chaplan et al., 1994; Ossipov et al., 1999). Animals displayed tactile and thermal allodynia. Spared nerve injury models involves the partial nerve injury- two of

three branches of the sciatic nerve- that reveals the opportunity of studying injured and uninjured neurons in the same DRG, but the ratio of injured neuron in the specific DRG should be estimated, and different levels of DRG may express different patterns of molecular expression. Moreover, the uninjured neuron distribution that innervates the sural territories of behavioral test should take note. Mechanical allodynia, hyperalgesia, and thermal hyperalgesia were published in SNI animals.

4.2 P2X3 immunoreactive neuron ratio in the ipsilateral lumbar DRG of SNI mice

In our study, unchanged P2X3 immunoreactive neuron ratio was quantitated between the control and surgical sides of L4 DRG in SNI mouse model one-week postsurgery. P2X3 (+) neurons were mainly expressed in small and medium sized neurons, while few percentages of large neurons were also discovered (Chen et al., 1995; Yun Sook Kim et al., 2008). Previous studies implied that P2X3 receptors were mostly projected into inner lamina II of dorsal horn (L. Vulchanova et al., 1998) that connected with small unmyelinated C fiber. The investigation in terms of P2X3 expression in large sized neurons was relative scarce and with less depth discussion. In inferior alveolar nerve injury of rat, 40% of large neurons were P2X3 immunoreactive in the trigeminal ganglia, and medium to large neurons were increased after nerve injury (J. Eriksson et al., 1998). About 30% of P2X3 (+) neurons in the trigeminal ganglia of rat were myelinated large sized neurons (V. Staikopoulos et al., 2007). The role of large sized neurons in P2X3 expression has been reported (Llewellyn Smith et al., 1998; Vulchanova et al., 1998; Xiang et al., 1998). And in another study, 4.8% of P2X3 (+) neurons in trigeminal ganglia of rat were large sized, with 7% of thin myelinated axon

that in the range of Aδ fiber size (Yun Sook Kim et al., 2008). This is familiar with our study in the DRG of SNI mice. P2X3 immunoreactivity was also discovered in large soma, although the quantities of large neuron in P2X3 (+) neuron population still remain an issue of debate, and the involvement of myelinated Aβ fiber in P2X3 signaling is possible.

P2X3 was highly colocalized with IB4 (+) neurons that were nonpeptidergic nocicpetive population, and rarely colocalized with CGRP (+) neurons that served as marker for peptidergic nociceptors, in the results of immunofluorescent staining using our rabbit- raised P2X3 antibody. About only 5% of P2X3 (+) neurons were also CGRP (+), which was consistent with previous reports (Yun Sook Kim et al., 2008). This was unchanged between contralateral and ipsilateral sides of DRG in SNI mice. However, in the double staining of P2X3 and IB4, the statistical analysis of P2X3 (+) neuron in IB4 (+) population showed a significant decline (~15%) in the surgical DRG. Given that unchanged P2X3 (+) neuron ratio and the combination of CGRP (+) and/or IB4 (+) neuron population in a DRG tissue in our study, it's likely to be a population shift of P2X3 (+) neurons after SNI. For the future plan it's intriguing to investigate the morphological distribution of P2X3 in SNI mouse model further and to search proper markers for immunofluorescent staining estimation. With the concerns of rabbit- raised P2X3 antibody, we also conducted the western-blot to compare with another antibody raised by different hosts (guinea- pig) preliminarily.

Controversial reports of P2X3 expression after nerve injury might be prompted by different injury models with variable mechanisms of neuropathic pain. In the mechanical injury models, different types of injury- degrees of impairment, location of injury, distribution of injured v.s intact nerves- could influence the overall outcome of P2X3 expression, and species difference could also be an issue. In a study of SNI rat

model, P2X3 mRNA was increased in lumbar DRG using in situ hybridization, while both down regulation and up regulation of the P2X3 receptor in sensory ganglia have been observed. Significant decrease of P2X3 mRNA in ATF3- immunoreactive neuron population together with the overall increase of P2X3 mRNA indicated increased P2X3 mRNA in the intact neuron population likely occurred (Kenzo Tsuzuki et al., 2001). The percentage of injured and intact neuron constitution in a DRG could be one reason of paradoxical findings of P2X3 expression. According to previous finding, L3 and L4 DRG both received abundant portion of neurons (~40%) from injured nerve in SNI, and the uninjured sural nerve fibers were traced back to L4 and L5 DRG, thus inferred the L4 is the most representative pair of DRG for estimating both injured and intact neurons in SNI mouse model. The expression level change of P2X3 in L5 is another important DRG level in the SNI mouse model to be investigated where the other half of pure intact sural spinal ganglia population located. The analysis of ATF3 co-expression with P2X3 population is also worth trying, for the purpose of analyzing injured and intact neuron populations and studying the relationship between P2X3 and ATF3.

In another study of SNI rat model, total expression of P2X3 in lumbar DRG was unchanged between the surgical and sham groups, while enhanced P2X3- mediated response and increased P2X3 in the plasma membrane revealed the possibility of membrane trafficking response to P2X3 sensitization (Yong Chen et al., 2005). Our SNI mouse model showed similar result. But we still require more evidence to confirm P2X3 sensitization or P2X3 distribution in the single sensory neuron. Since the SNI mice displayed mechanical allodynia, unchanged P2X3 expression level reveals that P2X3 might not be involved in this abnormal hypersensitivity. Time course of the dissected tissue could be another possible issue to be investigated. It is still uncertain about the initiation and maintenance stages of the mechanisms of P2X3 expression in

neuropathic pain pathology. In the researches of peripheral P2X3 expression using animal neuropathic pain models, the time points of dissected DRG tissue vary, therefore lead to a possible issue related to paradoxical P2X3 expression.

In order to obtain verified data of pain molecules expression in DRG using immunofluorescent staining, series of standard procedures are conducted strictly. Consistent, rigid and attentive technical procedures are important to minimize additional variables between individuals in in vivo studies.

4.3 Neuropathic pain behaviors in the ATF3-knockout mice

In our study, the ATF3-knockout mice displayed mechanical allodynia after SNI surgery, which were similar to the wild- type mice. The comparison of pain behavior between these two genotypes suggests that ATF3 may not play the primary role in the mechanism of this neuropathic pain sensation, but the importance of ATF3 still cannot be fully excluded. The control of ATF3-KO mice study should use the littermate wild-type individuals in the future to acquire identical genetic backgrounds between two genotypes as much as possible. To mimic the human chronic neuropathic pain states clinically, the observation time of pain behaviors in our neuropathic pain model should also be prolonged proportional to the animals' life span. Since the expression level of P2X3 in SNI-operated DRG was not significantly different from the control side, the relationship between P2X3 and ATF3 in neuropathic pain signaling still worth to be investigated, probably with different neuropathic pain models.

FIGURES & TABLES

Figure 1.

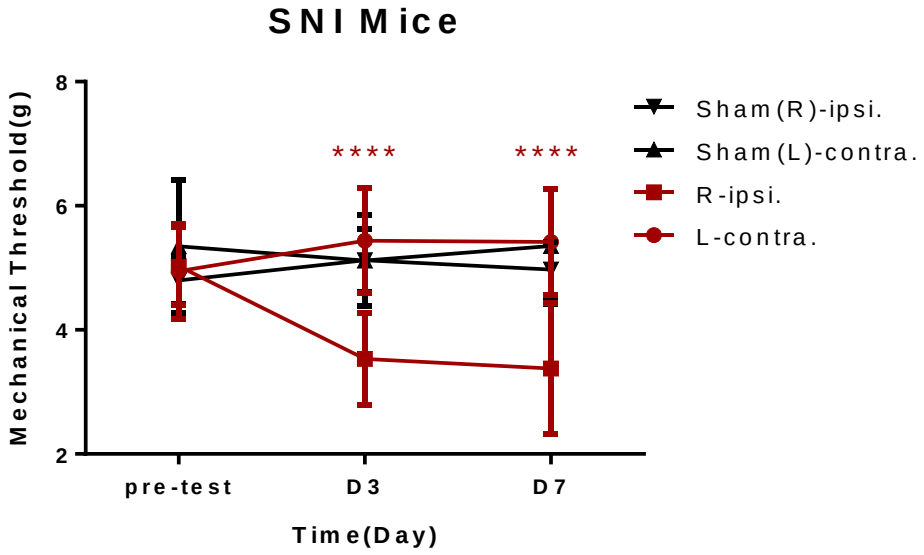


Fig.1 Reduced mechanical thresholds in mice after SNI surgery using dynamic plantar aesthesiometer. In the SNI group (red lines), the absolute values of mechanical threshold (g) on ipsilateral limbs were similar to contralateral limbs on pretest, and declined on D3 and D7 post-surgery ($p<0.0001$) significantly. In the sham group (black lines), the comparison of mechanical thresholds on both limbs have no significant difference. (Sample size: SNI= 29; sham= 6)

Figure 2.

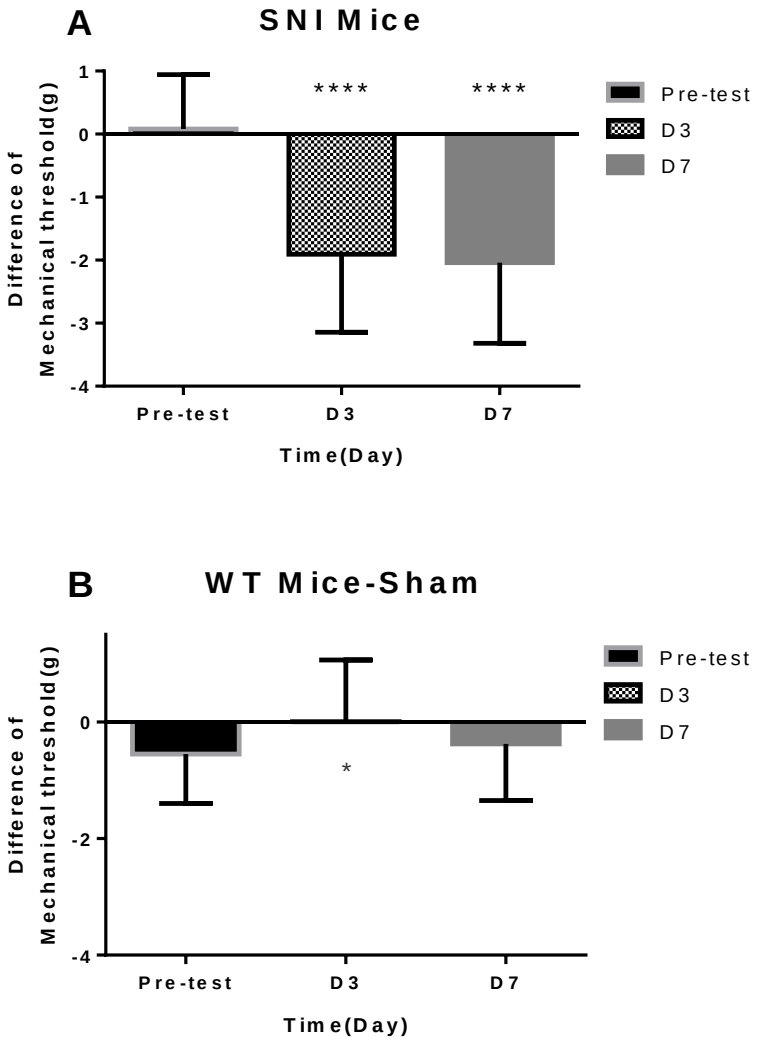
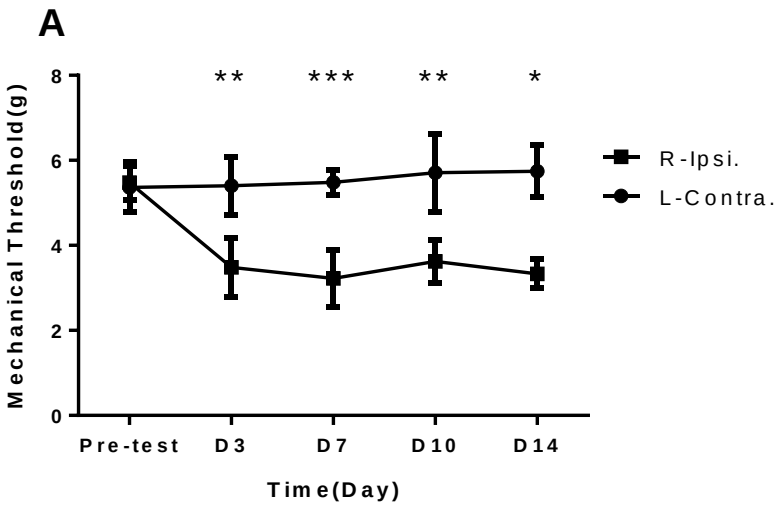

A
SNI Mice
Difference of Mechanical threshold(g)
1
0
-1
-2
-3
-4
****
****
Pre-test
D3
D7
Pre-test
D3
D7
Time(Day)
B
WT Mice-Sham
Difference of Mechanical threshold(g)
0
-2
-4
*
Pre-test
D3
D7
Pre-test
D3
D7
Time(Day)

Fig. 2 Differences of mechanical threshold in the mice after SNI surgery in the format of bar chart.

(A) In the SNI group, difference of mechanical threshold (ipsilateral side- contralateral side, unit: g) showed negative values on D3 and D7 with significant difference in comparison with pretest ($p < 0.0001$).

(B) In the sham group, $p<0.05$ on D3 compared to pretest ($p = 0.0169$, paired t-test), while on D7 the difference of mechanical threshold was similar to pretest, which was in line with our expectation. (Sample size: SNI= 29; sham= 6)

Figure 3.


A
**
***
**
*
8
6
4
2
0
Mechanical Threshold(g)
Pre-test
D3
D7
D10
D14
Time(Day)
R-Ipsi.
L-Contra.

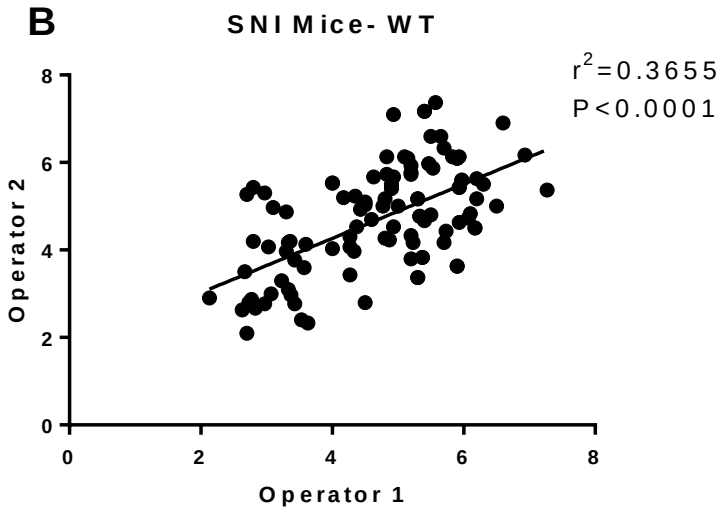
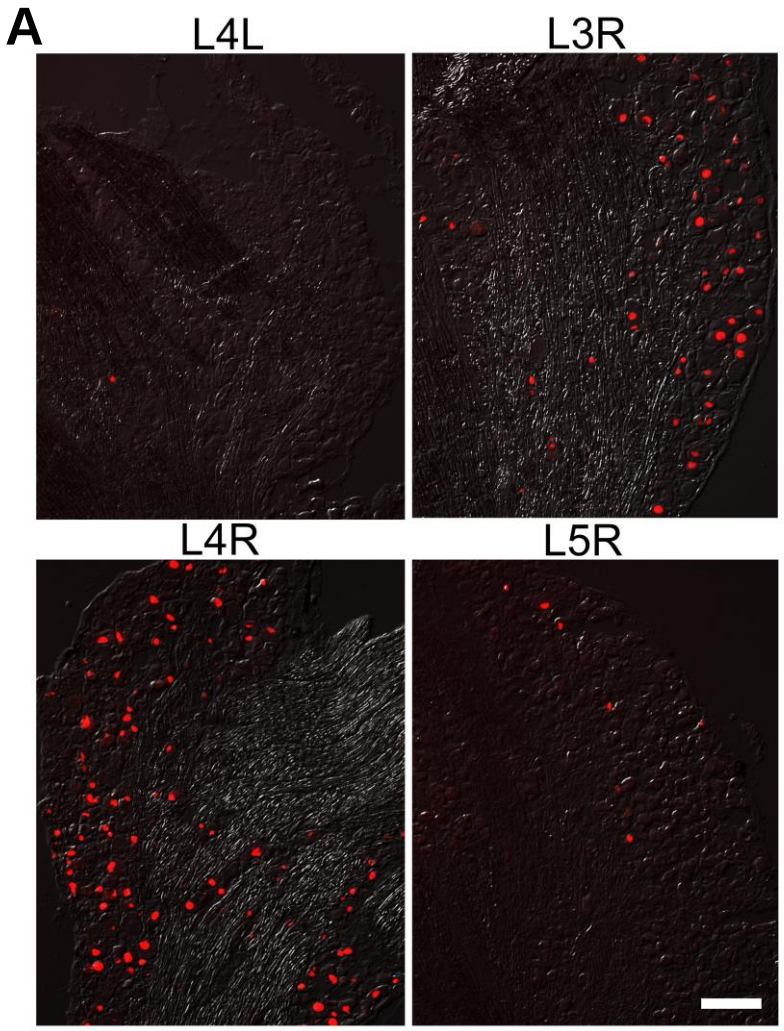

B
SNI Mice- WT
r2=0.3655
P<0.0001
8
6
4
2
0
Operator 2
0
2
4
6
8
Operator 1

Fig. 3 Persistence time of neuropathic pain behavior in SNI mice **(A)**, and the inter-operator correlation of the mechanical thresholds in each individual mouse **(B)**.

In figure 3A, reduced mechanical thresholds developed D3 post-surgery and persisted at least to D14. The difference of mechanical threshold on D3 to D14 showed significant difference in comparison with pretest ($p= 0.0035$ on D3; 0.0009 on D7; 0.0097 on D10; 0.0116 on D14, paired t- test). The mechanical thresholds on ipsilateral side were similar from D3 to D14 (sample size: SNI=5). In figure 3B, mechanical thresholds (g) tested by two independent operators were analyzed by linear regression and showed positive correlation ($r^2= 0.3655$, $p<0.0001$). (Sample size: 3A= 5; 3B= 14)

Figure 4.


A
L4L
L3R
L4R
L5R

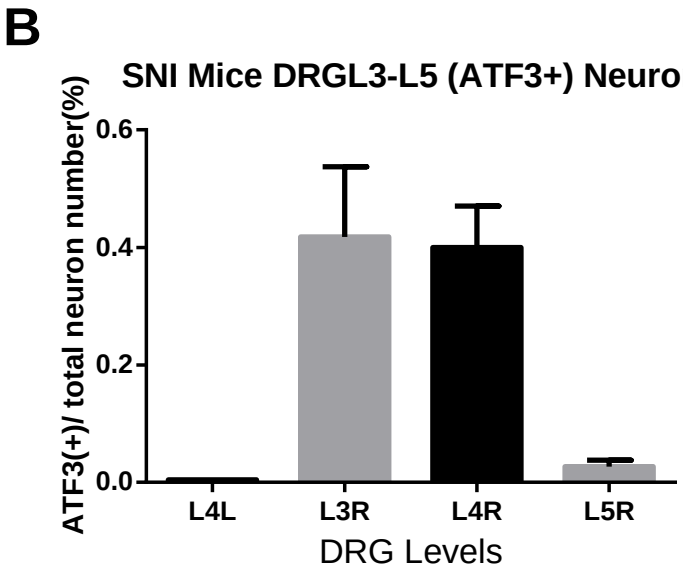
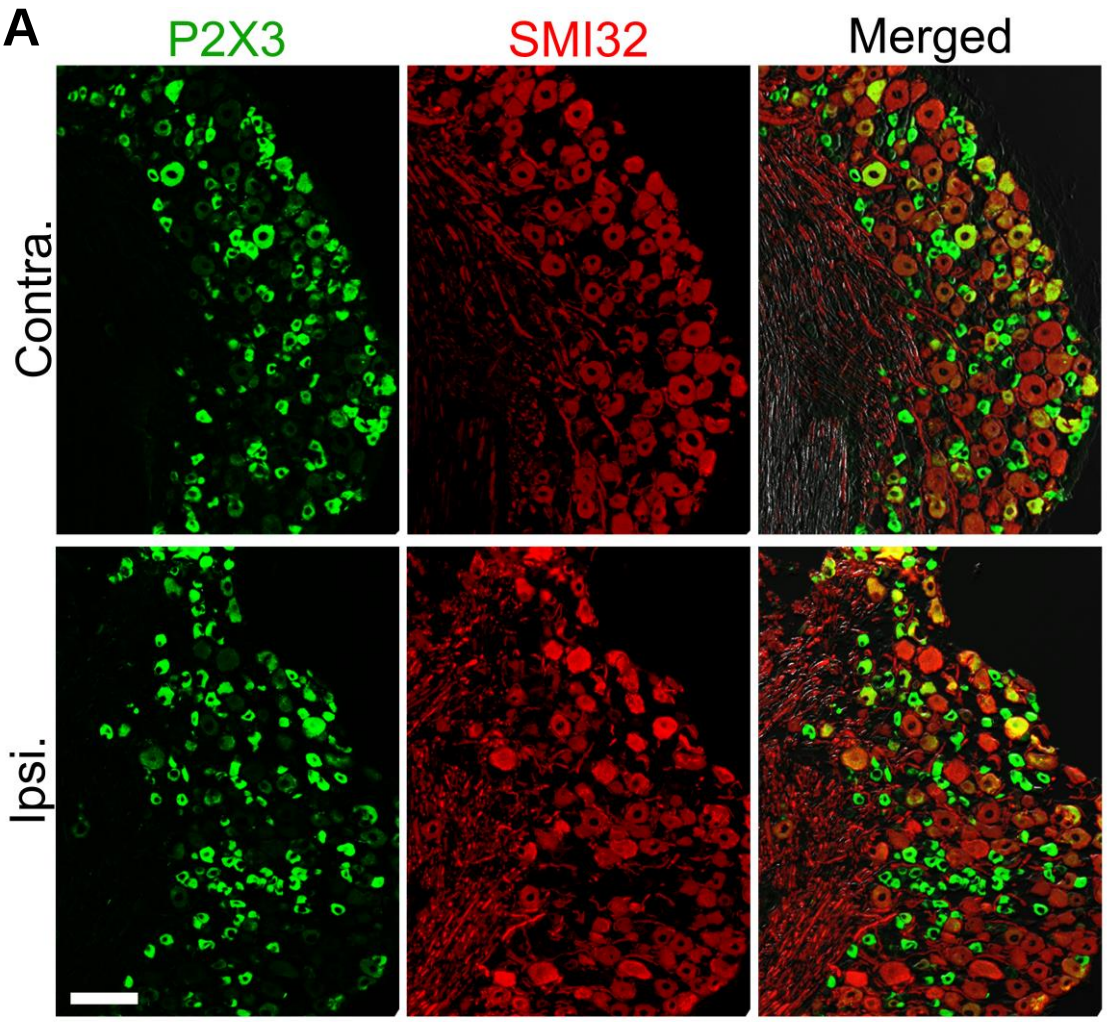

B
SNI Mice DRGL3-L5 (ATF3+) Neuron
ATF3(+)/ total neuron number(%)
0.6
0.4
0.2
0.0
L4L
L3R
L4R
L5R
DRG Levels

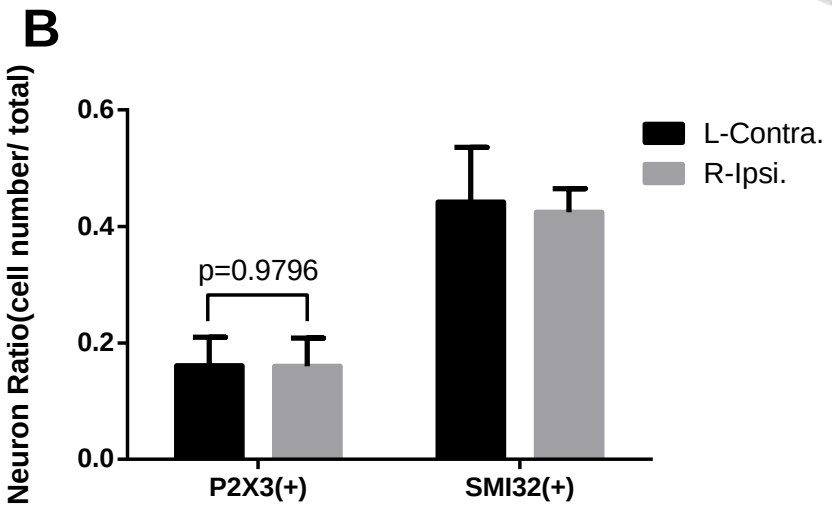
Fig. 4 Injured neuron distribution in L3-L5 DRG of mice sciatic nerve after SNI

(A) Immunofluorescent staining of ATF3 under, a nerve injury neuronal marker. Most of the injured tibial & common peroneal nerves are located in L3 and L4 DRG, whereas few ATF3 (+) neurons were found in L5 (scale bar= 100 μm).

(B) Quantitation of ATF3 (+) neuron ratio on ipsilateral DRG from L3 to L5 (L3= 41.8± 9.7%; L4= 40±5.7%; L5= 2.7±0.9%) (Sample size= 3).

Figure 5.




B
Neuron Ratio(cell number/ total)
0.6
0.4
0.2
0.0
p=0.9796
L-Contra.
R-Ipsi.
P2X3(+)
SMI32(+)

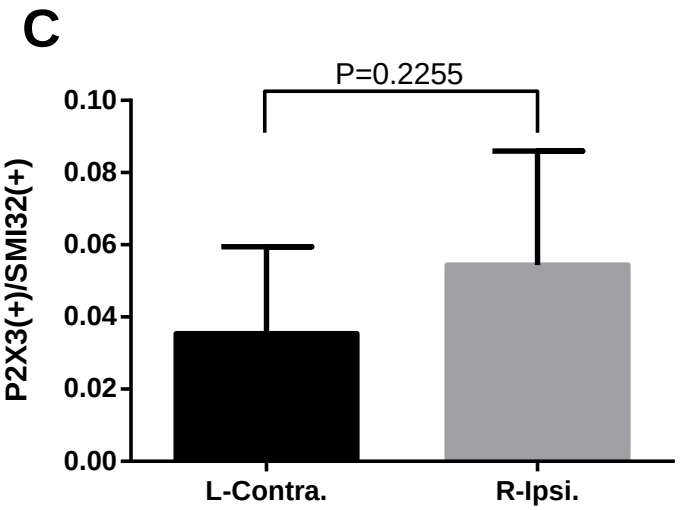

C
P=0.2255
P2X3(+)/SMI32(+)
0.10
0.08
0.06
0.04
0.02
0.00
L-Contra.
R-Ipsi.

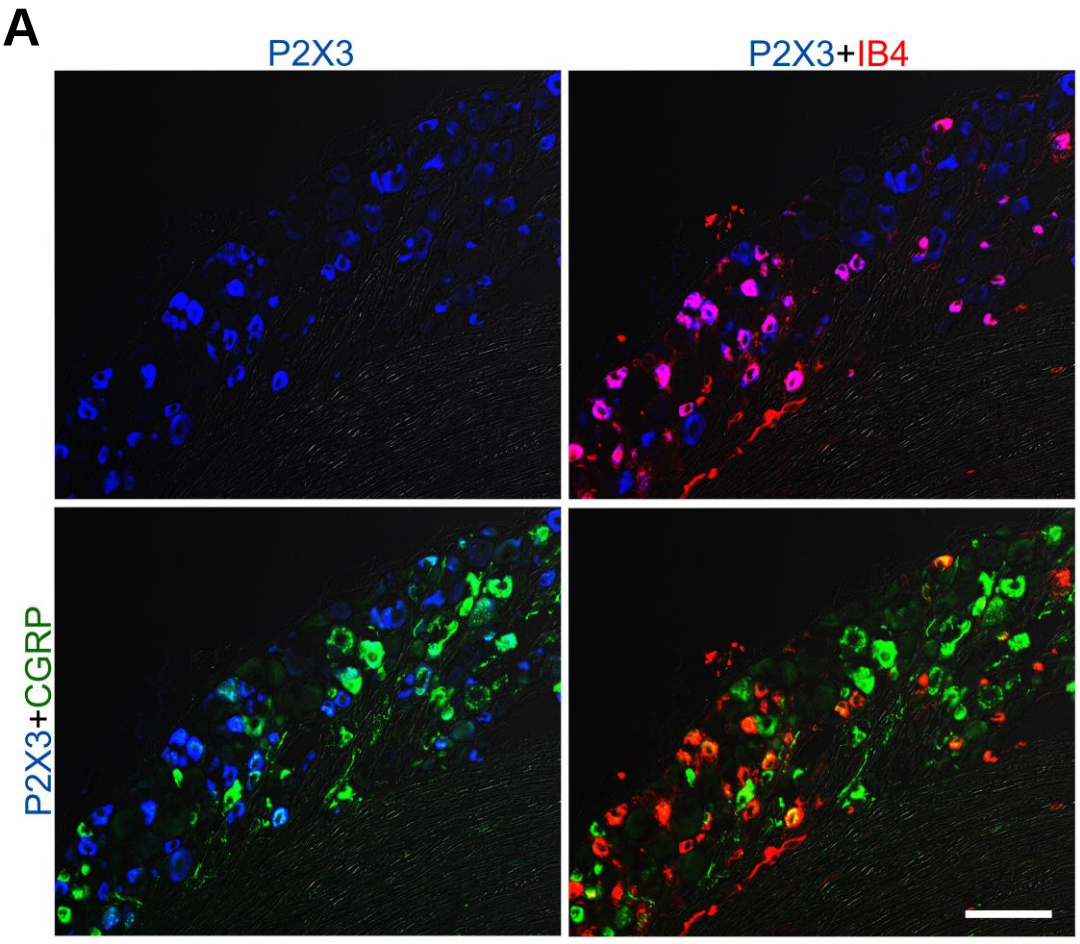
Fig. 5 P2X3 expression in the L4 DRG after SNI

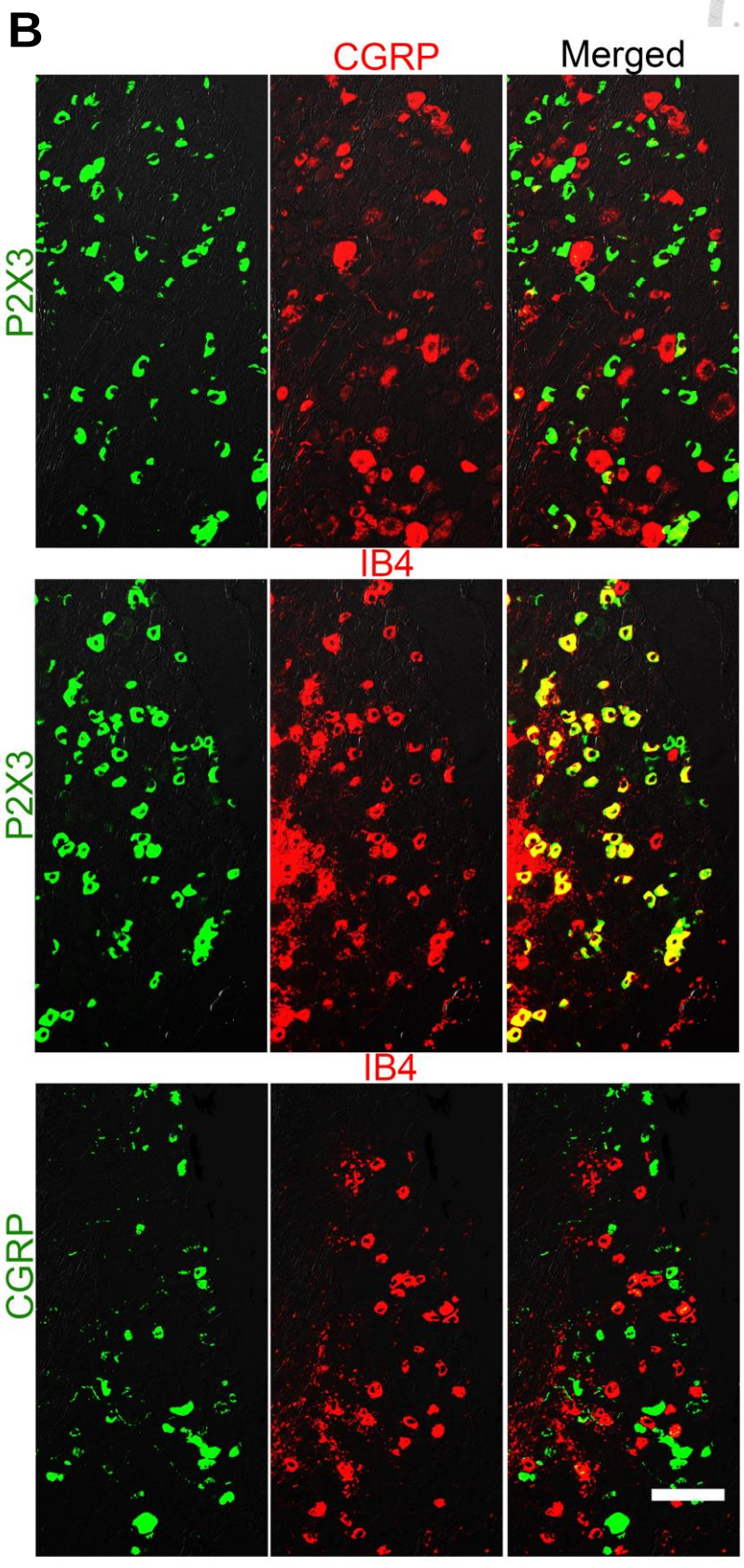
(A) Double staining of P2X3 (green) and SMI32 (red), a marker of large sized neurons. P2X3 was mainly expressed in small to medium neurons, while colocalization of SMI32-labeled large sized neurons was also observed. (scale bar= 100 μm)

(B) Quantitative data of Fig. 5A: P2X3 immunoreactive neuron ratio between contralateral and ipsilateral DRG was no significant difference (p= 0.9796, paired t-test).

(C) Estimation of P2X3 expression in the SMI32 (+) subgroup: P2X3 receptors were slightly increased in large sized neurons, while it doesn't showed statistical meaning (p=0.2255, paired t- test) (Sample size= 6).

Figure 6.




B
CGRP
Merged
P2X3
IB4
P2X3
IB4
CGRP

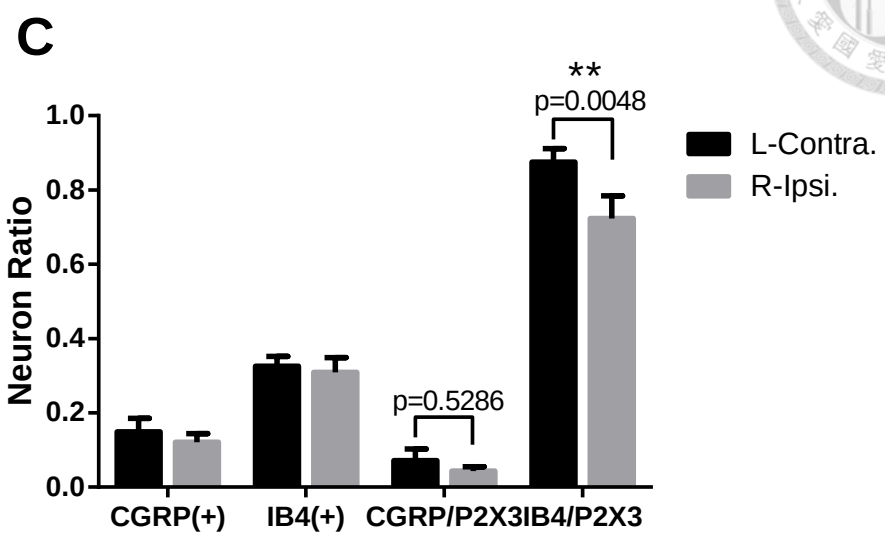


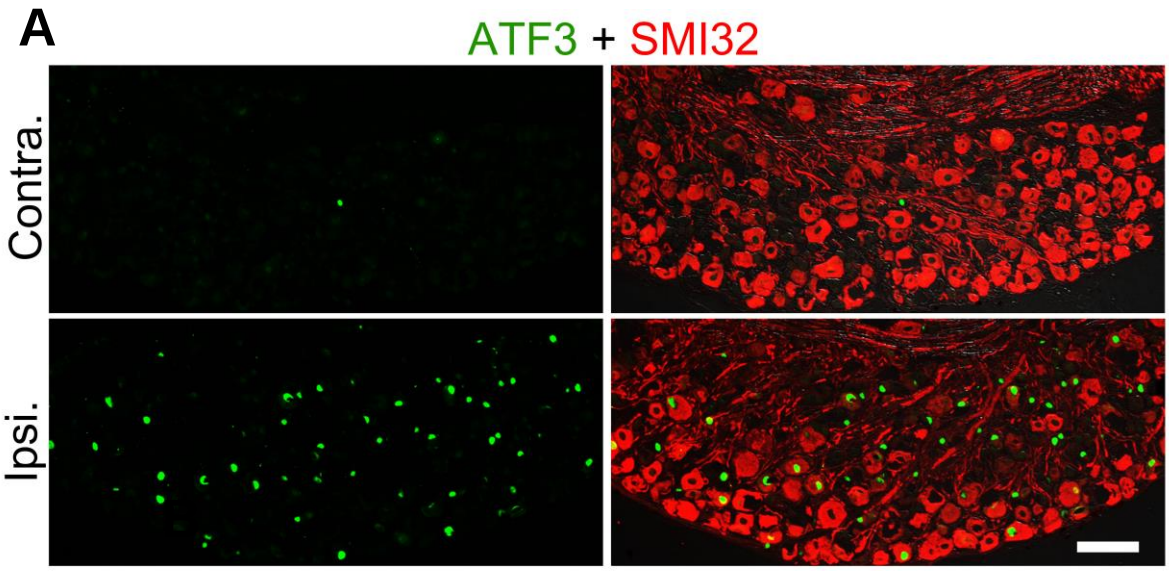
Fig. 6 Colocalization of P2X3 with IB4 and CGRP

(A) Triple staining of P2X3 (blue), IB4 (red), and CGRP (green). P2X3 immunoreactivity was highly colocalized with nonpeptidergic IB4 (+) neurons and rarely colocalized with CGRP that expressed in peptidergic subgroup. Merged photo of IB4 and CGRP showed scarce overlap (n=1).

(B) Double staining of P2X3+CGRP (n=4), P2X3+IB4 (n=5), and CGRP+IB4 (n=4) between contralateral and ipsilateral L4 DRG. (Scale bar in A and B= 100 μm)

(C) Quantitative data of fig. 6B: CGRP (+) neuron ratios, IB4 (+) neuron ratios, and CGRP/P2X3 neuron ratios were unchanged between contralateral and ipsilateral sides (CGRP (+)= 16.3± 8.14% v.s 14.29± 2.11%; IB4 (+)= 32.54± 2.64% v.s 30.87± 3.98%; CGRP/P2X3= 8.44± 6.6% v.s 6.04±0.84%, p=0.5286). IB4/P2X3 neuron ratios in the ipsilateral DRG were significantly declined (87.47± 3.67% v.s 72.29± 6.16%, p= 0.0048).

Figure 7.


A
ATF3 + SMI32
Contra.
Ipsi.

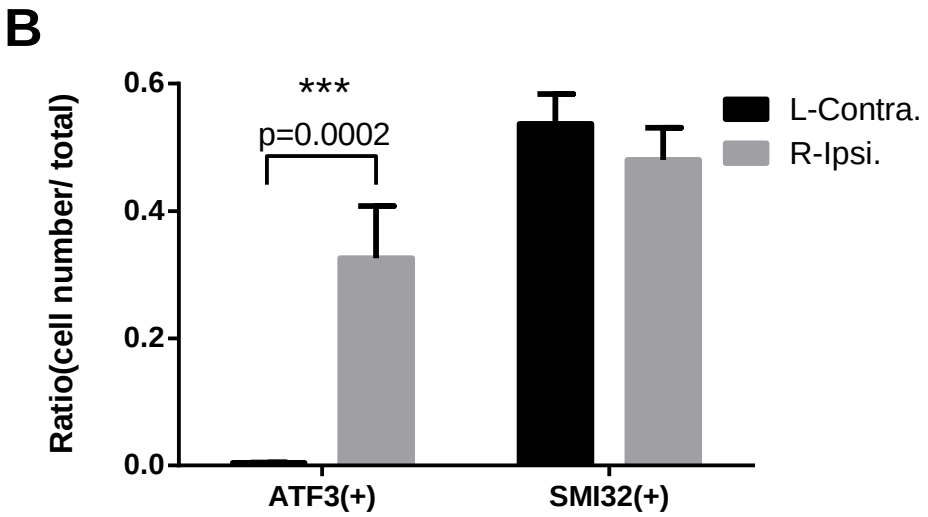

B
Ratio(cell number/ total)
0.6
0.4
0.2
0.0
***
p=0.0002
L-Contra.
R-Ipsi.
ATF3(+)
SMI32(+)

Fig. 7 Increased expression of ATF3 on ipsilateral DRG after SNI

(A) Double staining of ATF3 and SMI32: the ATF3 (+) neuron expression on the ipsilateral DRG was increased dramatically. (Scale bar= 100 μm)

(B) Quantitation of fig. 7A: ATF3 (+) neuron ratio between contralateral and ipsilateral sides= 0.44± 0.05% v.s 32.56±8.2%, p= 0.0002) (Sample size= 5)

Figure 8.

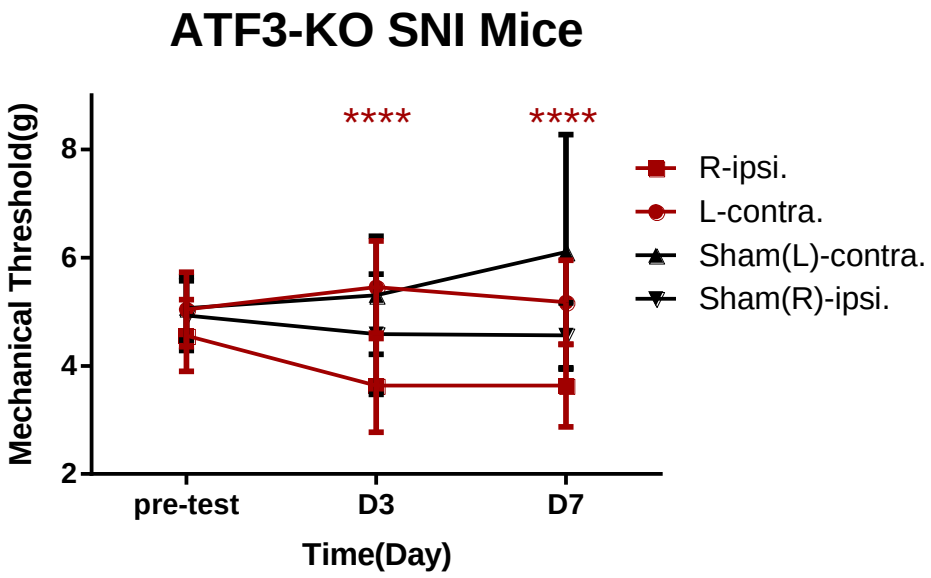

ATF3-KO SNI Mice
Mechanical Threshold(g)
8
6
4
2
****
****
pre-test
D3
D7
Time(Day)
R-ipsi.
L-contra.
Sham(L)-contra.
Sham(R)-ipsi.

Fig. 8 Reduced mechanical thresholds are also observed in ATF3-KO mice after SNI surgery, displayed in the format of absolute mechanical thresholds. The comparison of mechanical thresholds between contralateral and ipsilateral sides on D3 and D7 of SNI group (red lines) showed significant difference ($p < 0.0001$); while in the sham group (black lines), the comparison of mechanical thresholds on both limbs have no significant difference. (Sample size: SNI= 16; sham= 5)

Figure 9.

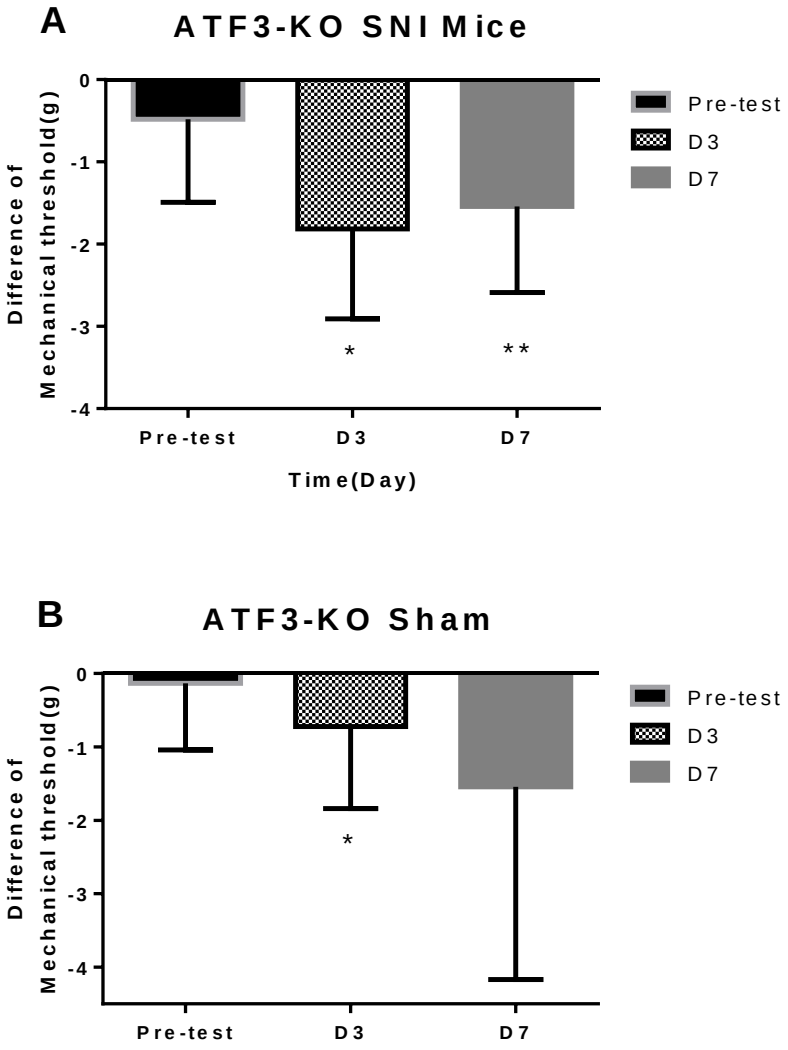
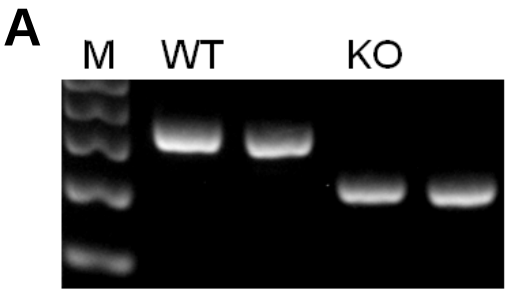

A
ATF3-KO SNI Mice
Difference of Mechanical threshold(g)
0
-1
-2
-3
-4
*
**
Pre-test
D3
D7
Pre-test
D3
D7
Time(Day)
B
ATF3-KO Sham
Difference of Mechanical threshold(g)
0
-1
-2
-3
-4
*
Pre-test
D3
D7
Pre-test
D3
D7

Fig. 9 Reduced mechanical thresholds in the ATF3-KO mice receiving SNI, displayed in the format of difference of mechanical threshold. **(A)** In the SNI group of ATF3-KO mice, difference of mechanical thresholds on D3 and D7 showed negative values and significantly different from the pretest (p value on D3= 0.0133, on D7= 0.0057, paired t-test). **(B)** In the sham group of ATF3-KO mice, the difference of mechanical threshold on D3 was negatively different from the pretest (p= 0.0277, paired t-test).

Figure 10.


A
M
WT
KO

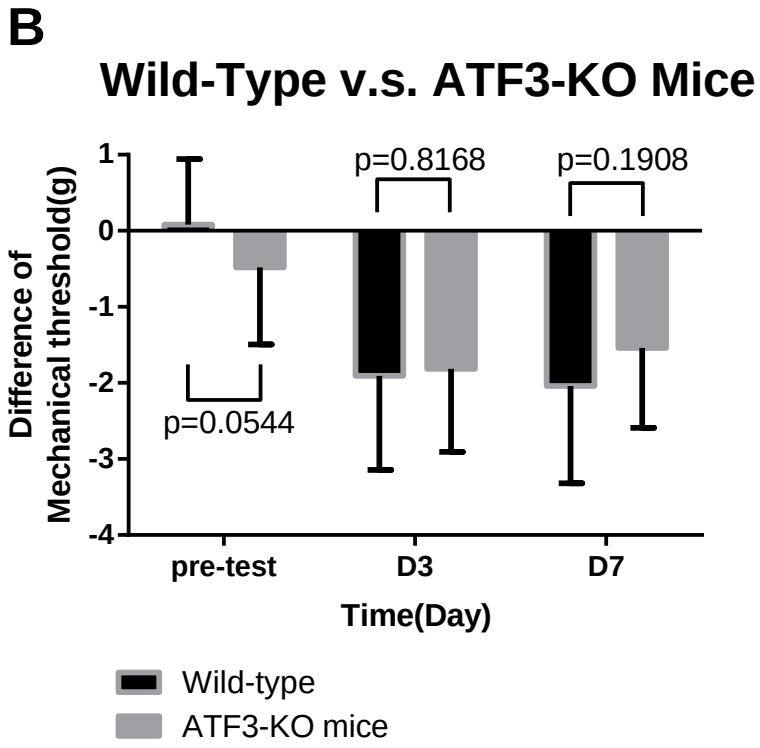

B
Wild-Type v.s. ATF3-KO Mice
Difference of Mechanical threshold(g)
1
0
-1
-2
-3
-4
p=0.8168
p=0.1908
p=0.0544
pre-test
D3
D7
Time(Day)
Wild-type
ATF3-KO mice

Fig. 10 Genotyping of the wild-type and ATF3-KO mice and the comparison of pain behavior after SNI in these two strains

(A) The KO allele was slightly shorter than the wild-type allele (236/ 329 bp).

(B) Comparison of difference of mechanical thresholds between wild-type and KO group on pretest, D3, and D7. (Sample size: wild-type= 29; ATF3-KO= 16) (p values on pretest= 0.0544, on D3= 0.8168, on D7= 0.1908; unpaired t-test)

TABLES

Table 1. Putative gene candidates involved in transcriptional regulation of P2X3 gene expression

Gene (Abbreviation) and Description
1. Adenosine A1 receptor (Adora1)
-Encodes a protein that exhibits G-protein beta/gamma-subunit complex binding, G-protein coupled adenosine receptor activity, G-protein coupled receptor binding; involved in activation of MAPKK activity, cognition; associated with abnormal brain ventricle and white matter morphology, diabetes mellitus, diabetic nephropathies
2. Aryl hydrocarbon receptor (Ahr)
-Binds 3-methylcholanthrene and other aromatic hydrocarbons; may play a role in bone formation; ligand-activated sequence-specific DNA binding RNA polymerase II transcription factor activity;sequence-specific DNA binding transcription factor activity
3. V-akt murine thymoma viral oncogene homolog 1 (Akt1)
-Encodes a protein that inhibits JUN kinase activation and mediates inhibition of apoptosis; ATP binding activity
4. Acid-sensing (proton-gated) ion channel 1 (Asic1)
-Forms a proton-gated ion channel that may play a role in sensory neuron function and pain perception
5. Activating transcription factor 1 (Atf1)
-With RNA polymerase II distal enhancer sequence-specific DNA binding transcription factor activity
6. Activating transcription factor 2 (Atf2)
- Transactivates tyrosine hydroxylase transcription; may be involved in regulation of catecholamine synthesis during neural development
7. Activating transcription factor 3 (Atf3)
-It's a stress-inducible gene and encodes a member of the ATF/CREB family of transcription factors. Its expression is associated with nerve injury neuronal marker and sequence-specific DNA binding transcription factor activity.

Gene (Abbreviation) and Description
8. B-cell CLL/lymphoma 2 (Bcl2)
-An anti-apoptotic protein; involved in inhibiting cell death in many different cell types; sequence-specific DNA binding and transcription factor binding activity
9. Brain-derived neurotrophic factor (Bdnf)
-Plays a role in the development of hippocampal long term potentiation; involved in regulation of synaptic plasticity, axon guidance, and dendrite development
10. Clock circadian regulator (Clock)
-Encodes a protein that plays a central role in the regulation of circadian rhythms; with sequence-specific DNA binding transcription factor activity; polymorphisms in this gene may be associated with behavioral changes, obesity, and metabolic syndrome
11. cAMP responsive element binding protein 1 (Creb1)
-Binds the cAMP response element in many gene promoters and regulates transcription; with RNA polymerase II activating transcription factor binding activity
12. cAMP responsive element binding protein 5 (Creb5)
-RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity involved in positive regulation of transcription
13. CREB binding protein (Crebbp)
-A transcriptional co-activator; has an important role in hormone-dependent female sexual behavior and long term memory; with RNA polymerase II transcription factor binding and core promoter proximal region sequence-specific DNA binding activities
14. cAMP responsive element modulator (Crem)
-Encodes a protein that exhibits sequence-specific DNA binding transcription factor activity
15. CREB regulated transcription coactivator 2 (Crtc2)
-Encodes a protein that exhibits cAMP response element binding protein binding; involved in positive regulation of CREB transcription factor activity
16. Dipeptidylpeptidase 4 (Dpp4)
-Also known as adenosine deaminase complexing protein 2 or CD26, is an antigenic enzyme expressed on the surface of most cell types and is associated with immune regulation, signal transduction, apoptosis, and abnormal pain threshold

Gene (Abbreviation) and Description
17. Estrogen receptor 1 (Esr1)
-Acts as a transcriptional activator when bound to estrogen with estrogen-activated sequence-specific DNA binding RNA polymerase II transcription factor activity; may play a role in myocardial regulation
18. Estrogen receptor 2 (ER beta) (Esr2)
-Binds estrogen and mediates transcriptional activation with sequence-specific DNA binding transcription factor activity
19. Fibroblast growth factor 2 (Fgf2)
-Activates the MAP kinase signaling pathway; plays a role in synaptic transmission; induces cell proliferation; involves in nerve growth factor receptor binding and gilal cell differentiation
20. FBJ osteosarcoma oncogene (Fos)
-An immediate early gene encoding a nuclear protein involved in signal transduction, with sequence-specific DNA binding transcription factor activity
21. Glial fibrillary acidic protein (Gfap)
-Intermediate filament protein specific for astrocytes
22. Heme oxygenase (decycling) 1 (Hmox1)
-Catalyzes the oxidative cleavage of heme to biliverdin, with negative regulation of DNA binding
23. Iroquois homeobox 6 (Irx6)
-IRX6 appears to play multiple roles during pattern formation of vertebrate embryos with sequence-specific DNA binding transcription factor activity
24. Jun proto-oncogene (Jun)
-Acts as a protooncogene with sequence-specific DNA binding transcription factor activity
25. Mitogen activated protein kinase 14 (Mapk14)
-Involved in intracellular signalling, inhibition of apoptosis and gene activation, with ATP binding and MAP kinase activity
26. Matrix metallopeptidase 2 (Mmp2)
-Plays important roles in wound healing, angiogenesis, platelet aggregation, and tumor metastasis; involves in peripheral nervous system axon regeneration

Gene (Abbreviation) and Description
27. Notch 1 (Notch1)
-Encodes a transmembrane receptor that involved in cell-cell interactions important for development and pattern formation; with RNA polymerase II transcription factor binding transcription factor activity involved in positive regulation of transcription
28. Proopiomelanocortin (Pomc)
-Involves in neuropeptide signaling pathway
29. Peroxisome proliferator-activated receptor gamma (Pparg)
-Encodes a ligand-activated transcription factor; mediates expression of genes involved in lipid metabolism; involves in Huntington disease pathway
30. Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (Ppargc1a
-Encodes a nuclear receptor coactivator with sequence-specific DNA binding transcription coactivator activity; may mediate glucose and lipid homeostasis
31. Ras-related C3 botulinum toxin substrate 1 (Rac1)
-Rac1 is a small signaling G protein (more specifically a GTPase); involved in axon guidance and cerebral cortex radially oriented cell migration
32. REX1, RNA exonuclease 1 homolog (S. cerevisiae) (Rexo1)
-Encodes a protein that exhibits exonuclease activity; nucleic acid binding activity; participates in ribosome biogenesis pathway
33. RNA exonuclease 2 (Rexo2)
-Encodes a protein that exhibits 3'-5' exonuclease activity; nucleic acid binding activity
34. REX4, RNA exonuclease 4 homolog (S. cerevisiae) (Rexo4)
-Encodes a protein that exhibits exonuclease activity; nucleic acid binding activity
35. Reticulon 1 (Rtn1)
-Encodes a protein that displays differential expression in response to ethanol during embryonic brain development; may be regulated by thyroid hormone; involved in neuron differentiation
36. Sigma non-opioid intracellular receptor 1 (Sigmar1)
-Mediates nerve growth factor induced neurite sprouting; may play a role in neuronal response to antidepressants; involved in nervous system development, opioid receptor signaling pathway, regulation of neuron apoptotic process

Gene (Abbreviation) and Description
37. Solute carrier family 7 (anionic amino acid transporter light chain, xc-system), member 11 (Slc7a11)
-Encodes a protein that exhibits amino acid transmembrane transporter activity; involved in brain development, lens fiber cell differentiation, response to nicotine, detection of temperature stimulus involved in sensory perception of pain
38. Ssignal transducer and activator of transcription 3 (acute-phase response factor) (Stat3)
-Encodes a transcription factor that plays a role in induction of gene expression during acute phase response
39. Transforming growth factor, beta 2 (Tgfb2)
-Encodes a secreted protein that performs many cellular functions, including the control of cell growth, cell proliferation, cell differentiation and apoptosis; involved in embryonic neurocranium morphogenesis, axon guidance
40. Tumor necrosis factor (Tnf)
-Acts as a cytokine; binds TNF receptors; plays a role in regulation of cell proliferation, induction of apoptosis, and inflammatory response, with transcription regulatory region DNA binding activity
41. TOX high mobility group box family member 3 (Tox3)
-Encodes a transcription factor containing HMG-box DNA-binding domains which function to modify chromatin structure; associated with negative regulation of neuron apoptotic process
42. Tumor protein p53 (Tp53)
-p53 is a DNA-binding protein containing transcription activation, DNA-binding, and oligomerization domains. It is postulated to bind to a p53-binding site and activate expression of downstream genes that inhibit growth and/or invasion, and thus function as a tumor suppressor
43. Tumor susceptibility 101 (Tsg101)
-Encodes a protein that may play a role in cell growth and differentiation and act as a negative growth regulator, with transcription corepressor activity

Gene (Abbreviation) and Description
44. Uncoupling protein 1 (mitochondrial, proton carrier) (Ucp1)
-Encodes a protein that plays a role in heat production by uncoupling oxidative phosphorylation from the respiratory chain
45. X-box binding protein 1 (Xbp1)
-Acts as a transcription factor that regulates MHC class II genes by binding to an X-box promoter element; agents that cause the unfolded protein response upregulate transcription
46. Exportin 1 (Xpo1)
-Binds human T-cell leukemia virus type 1 Rex protein and exports it to the cytoplasm
47. Zinc finger protein 42 (Zfp42)
-Rex1 (Zfp42) is a known marker of pluripotency, and is usually found in undifferentiated embryonic stem cells
48. Zinc finger protein 42-like (Zfp42l)
-Encodes a protein that exhibits metal ion binding, nucleic acid binding activity; involved in gonad development and in utero development
49. Zinc finger protein 394 (Zfp394)
-Function with sequence-specific DNA binding transcription factor activity

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