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論文題目

足三里穴位注射第一型瞬態電位類香草受器(TRPV1)促效劑而非第四型瞬態電位類香草受器(TRPV4)與第三型酸敏感離子通道(ASIC3) 促效劑能在小鼠產生類針灸止痛效應: 第一型瞬態電位類香草受器可能是針灸刺激的感應通道?

TRPV1 but not TRPV4 and ASIC3 agonist injection replicates acupuncture-like analgesic effect at Zusanli acupoint (ST36) in mice: Possible role of mechanosensitive TRPV1 as "acupuncture responding channel"?

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論文指導教授推薦書

針灸研究所，吳 書 毅 君所提之論文 足三里穴位注射第一型瞬態電位類香草受器(TRPV1)促效劑而非第四型瞬態電位類香草受器(TRPV4)與第三型酸敏感離子通道(ASIC3)促效劑能在小鼠產生類針灸止痛效應：第一型瞬態電位類香草受器可能是針灸刺激的感應通道？(題目)，係由本人指導撰述，同意提付審查。

指導教授 林以文 (簽章)

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中國醫藥大學碩士班研究生
論文口試委員審定書

針灸研究所，吳書毅君所提之論文足三里穴位注射第一型瞬態電位類香草受器(TRPV1)促效劑而非第四型瞬態電位類香草受器(TRPV4)與第三型酸敏感離子通道(ASIC3)促效劑能在小鼠產生類針灸止痛效應：第一型瞬態電位類香草受器可能是針灸刺激的感應通道？(題目)，經本委員會審議，認為符合碩士資格標準。

論文口試委員會　　委員 林以文 （簽章）

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中華民國 102 年 6 月 4 日

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第一型瞬態電位類香草受器可能是針灸刺激的感應通道?

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關鍵字: 第一型瞬態電位類香草受器;第四型瞬態電位類香草受器;第三型酸敏感離子通道;針灸;力學傳遞

針灸是一種在穴位做力學刺激的治療。已經有許多的研究指出針灸機轉是藉由鴉片類胜肽(opioid peptide)與腺嘌呤核苷(adenosine)進行神經調控，但儘管如此我們仍對於物理性刺激如何變成神經訊號有許多不解。第一型和第四型瞬態電位類香草受器(TRPV1 與 TRPV4)與第三型酸敏感離子通道(ASIC3)已被認定可能是機械式感應通道(mechanosensitive channel)。這篇論文的目的在於探討，這三個通道在足三里穴位的角色。首先，從足三里與偽穴位(sham point)的組織採樣中發現，這三個通道在足三里有較多的表現。第二，從免疫螢光染色發現這三個通道在神經細胞與非神經細胞皆有表現。第三，將這三個通道的促效劑(agonist)注射到有發炎性疼痛小鼠的足三里內，發現只有TRPV1 的促效劑(辣椒素,capsaicin)可以產生跟針灸一般的止痛效果。由於機械力感應已經被證實可以藉由鈣離子波(calcium wave propagation)將訊號由非神經細胞傳遞到神經纖維。因此，針灸的感傳也許包含了神經細胞發起與非神經細胞發起的兩種感知途徑。而鈣離子波的相關蛋白皆與TRPV1 表現在相同的解剖層的結果也支持了上述想法。這篇論文的結果可以使我們更清楚針灸的感應介面，包含物理性刺激如何被接受而又轉換為生物訊號。

TRPV1 but not TRPV4 and ASIC3 agonist injection replicates acupuncture-like analgesic effect at Zusanli acupoint (ST36) in mice: Possible role of mechanosensitive TRPV1 as "acupuncture responding channel"?

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Key words: TRPV1; TRPV4; ASIC3; Acupuncture; Mechanotransduction

Acupuncture is a therapy that involves applying mechanical stimulation to acupoints using needles. Although many authors have reported acupuncture analgesia related to neural regulation by opioids or adenosine, little is known about how physical stimulation is turned into neurological signaling. The transient receptor potential vanilloid receptors 1 and 4 (TRPV1 and TRPV4) and the acid-sensing ion channel 3 (ASIC3) are regarded as mechanosensitive channels. This thesis aimed to clarify their roles at the Zusanli acupoint (ST36). First, tissues from ST36 and the sham point were collected. Then an abundance of channels in ST36 were demonstrated. Second, the immunofluorescence microscopic slides showed the channels were expressed in neural and non-neural cells. Third, agonists of channels were injected into ST36 and tested in a mouse inflammatory pain model. Only capsaicin, a TRPV1 agonist, replicated the analgesic effect of acupuncture. Mechanical sensing was reported by non-neural cells and processed to nerve terminals via calcium wave propagation (CWP). It is likely that both neural and non-neural cell initiated sensing pathways are triggered during acupuncture. This was supported by co-expression of TRPV1 and CWP components in the same anatomical layers. The results may lead to a better understanding of the interface between physical stimulation by acupuncture and biological signaling to neurons.

Chapter 1 : Introduction

Acupuncture is an ancient therapy used for more than two thousand years by traditional Chinese medicine (TCM) doctors to treat disorders. However, it was until recent decades that acupuncture gain its world-wide acknowledgement by western societies.[1] According to ancient TCM philosophy, disorders were caused by disruption of qi (氣) in merdians (acupuncture channels, 經絡) or visceral organs.[2] Therefore the principal treatment strategy of acupuncture is to smoothen the flow of qi and regain balance in the body. This is done by inserting needles into acupoints followed by in traditionally manual manipulation (manual acupuncture, MA) or newly developed electrostimulation (electroacupuncture, EA). After introducing acupuncture to the west, many of its therapeutic effect was demonstrated in animal models, like in epilepsy,[3] stroke,[4] and pain treatment.[5-7] Also, its efficacy was confirmed in clinical trials and approved its benefits particularly in pain management.[8-13] But even with strong evidences in treating disorders, the biological mechanism of acupuncture treatment remained to be answered.

Many authors have reported the involvement of endogenous opioids and the participation of descending inhibitory pathways.[14-17] Recently, Goldman et al. demonstrated localized ATP release at acupoint after MA.[18,19] ATP is then metabolized to anti-nociceptive adenosine by maybe prostatic acid phosphatase in muscles, and resulting in analgesia. However, it remains unknown how mechanostimulation from acupuncture induce ATP release and neural stimulation.

This thesis suggests that mechanosensitive channels are involved in the reception of mechanostimulation by acupoints during acupuncture. The transient receptor potential vanilloid receptors 1 and 4 (TRPV1 and TRPV4) and the acid-sensing ion channel 3 (ASIC3) are mechanosensitive channels related to local ATP release in various tissues.[20-24] Also, MA effects are

blocked by gadolinium, a non-selective mechanosensitive channel blocker that also blocks TRPV1, TRPV4 and ASIC3.[25] Considering their mechanosensitivity and the role played in stimulation induced ATP release, it is highly possible that these channels participate in acupuncture sensing.

It is noteworthy that local ATP released is related to the intercellular purinergic signaling called calcium wave propagation (CWP). The phenomenon is universal and is reported among glia,[26] salivary glands,[27] nephrons,[28] fibroblasts,[29] etc. In keratinocytes[30] and subepithelial fibroblasts[29] of villi, it has been proposed that cells respond to ATP release and send signal to neurons via CWP after stimulation of non-neural cells. Since mechanosensitive channels are related to ATP release and the occurrence of CWP during acupuncture in non-neural cells was recently reported.[31] It would be interesting to determine if mechanosensitive channels and molecular components for CWP co-localize in the same anatomical layer of acupoint. If so, then it is possible that acupuncture can be sensed by mechanosensitive channels from non-neural cells and pass on signaling to neuron by ATP induced CWP.

In this thesis, hypothesis was made that during manual acupuncture, mechanosensitive channels participate in both neural and non-neural cell initiated sensing pathways. This was first demonstrated by the abundant mechanosensitive channels expression at neural and non-neural tissues of acupoint followed by the replication of the acupuncture analgesic effect after injecting agonist of the channels into acupoint. Finally, abundant co-expression of pannexin 1, connexin 43, P2Y1, and P2Y2 with mechanosensitive channels at acupoint was demonstrated. This implies occurrence of CWP after channel stimulation. With these results, a better picture of the interface between physical stimulation by acupuncture and biological signaling to the nervous system is provided.

Chapter 2 : Literature Review

2.1 Mechanisms of acupuncture analgesia

There are many proposed mechanisms regarding to acupuncture analgesia. Among which, the most acknowledged is the neural mechanism.[32] It is proposed since acupuncture analgesia can be blocked if procaine is injected into acupoint in advance of acupuncture. The neural regulation mechanism can be divided as the central or the peripheral (local) mechanism depending on the site of regulation.[17]

Furthermore, the central regulation can be divided into the spinal mechanism and the supraspinal mechanism. For the spinal mechanism, it is believed that the acupuncture stimulation gives inhibitory regulation to noxious stimulation-induced impulses. This was demonstrated in cat that electroacupuncture at ST36 produce the inhibitory post-synaptic potentials (IPSPs) and long-lasting membrane hyperpolarization in nociceptive spinal dorsal horn neuron.[33] For the supraspinal mechanism, it is believed that acupuncture impulse transfer signals from spinal dorsal horn through ventrolateral funiculus to pain regulating areas in the brain and then modulate pain impulse after transferring inhibitory impulse by way of descending inhibitory pathway to pain related lamina in dorsal horn. This hypothesis of acupuncture analgesia was strengthen by experiments demonstrated that the acupuncture effect was attenuated after creating section or lesion to the corresponding ascending and descending tracts.[34,35] During the past decades, many nuclei in the brain were identified to participate in acupuncture analgesia,[17] these include, RVM (mainly NRM), periaqueductal gray (PAG), locus coeruleus (LC), arcuate nucleus (Arc), preoptic area (Po), centromedian nucleus (CM), nucleus submedius (Sm), anterior pretectal nucleus (APtN), habenular nucleus (Hab), nucleus accumbens (Ac), caudate nucleus (Cd), septal area (Sp), amygdale, anterior

cingulated cortex (ACC), and hypothalamic paraventricular nucleus (PVH). Among them, the Arc-PAG-NRM-spinal dorsal horn pathway and the opioid receptors seem to play a vital role during acupuncture analgesia.

Recently, a localized mechanism for acupuncture analgesia was raised by Goldman *et al.*[19] They demonstrated with microdialysis and high performance liquid chromatography (HPLC) that during manual acupuncture (MA), ATP was release abundantly at the acupoint after manipulation. This localized ATP release was particularly evident in the acupoint compared to the non-acupoint. They also showed that ATP was later degraded to pain relieving adenosine by maybe prostatic acid phosphatase and resulted in local analgesia. The participant of adenosine is further supported by their use of adenosine A1 receptor knock-out mice. After inducing inflammatory pain or neuropathic pain on the adenosine A1 receptor knock-out mice, MA on the adenosine A1R knock-out mice failed to repeat analgesia as in wild-type mice. This is a strong evidence for involvement of adenosine A1 receptor during acupuncture analgesia. Works from Goldmen *et al*. showed that ATP was released after manipulation on acupoint. After then, ATP was dephosphated to adenosine and result in acupuncture analgesia by activating inhibitory adenosine A1 receptor. Their study sure is a milestone in acupuncture research, but questions remained to be answered as how ATP is released during MA and why in inflammatory pain model acupuncture-induced inhibitory effect on innervated nerve of ST36 (deep peroneal nerve) is related to desensitize on innervated nerve of paw (tibia nerve)?

2.2 Properties of TRPV1 channel

The transient receptor potential vanilloid 1 (TRPV1) is a nonselective cation permeable channel belong to the transient receptor potential vanilloid family. It can react to various stimulations including capsaicin, heat (>43°C), low pH, voltage, and endogenous lipid.[36] Although TRPV1 is permeable to cations as sodium, potassium, and calcium, TRPV1 showed preference for calcium. The TRP family channels are composed of six transmembrane segments (S1-6) with a pore forming loop between S5 and S6. Like most TRPV channels, a six ankyrin repeats is found at its cytosolic N-terminus, which is the site for phosphorylation and to sensitize the channel. [37]

Before first recognition, its specific agonist, capsaicin, has long been used as reagent to activate the pain-related C-fibers. Interestingly, after activation of C-fibers, an initial pain sensation is caused. But when capsaicin is given repeatedly or at a high dose, within seconds, an inhibitory effect named conduction analgesia or desensitization is induced.[38,39] The conduction analgesia is generated by calcium influx into cell which interact with calmodulin to cause dephosphorylation of TRPV1[40,41] and by tonic inhibition from PIP2.[42,43] However, the conduction analgesia is not the only mechanism for analgesia by capsaicin; another mechanism called noxious stimulus-induced analgesia (NSIA) also participated in capsaicin induced analgesia and will be discussed later.

It is worth mentioning that ATP released to surrounding was reported after activating TRPV1 on urothelial cells.[20,21] It is yet not certain of the mechanism for ATP release after TRPV1 activation. Nonetheless, the mechanism for ATP release after mechanical stimulation was reported for another TRPV channel, TRPV4. In the case of TRPV4, Rho kinase was showed to participate after channel activation and trigger ATP release by hemichannel pannxin 1.[22]

2.3 Properties of TRPV4 channel

The transient receptor potential vanilloid 4 (TRPV4) is another mechanosensitive channel from the TRPV channel family. It is structurally similar to TRPV1 and also has preference to permit calcium.[44] Other than heat (>24-27˚C), TRPV4 can be activated by chemicals as 4α-PDD, GSK1016790A, epoxyeicosatrienoic acids, bisandrographolide as well as physical stimulations such as osmolality change and mechanostimulation. TRPV4 was detected in various diameters of neurons in the DRG, therefore the expression is not restricted to small diameter fibers as C-fibers.[45]

Similar to TRPV1, TRPV4 is related to local ATP release from cell after mechanical stimulations.[20,22,23] As reported by Seminario-Vidal et al. in airway epithelial cells, Rho kinase activation occurred after TRPV4 activation and trigger ATP release by hemichannel pannxin 1.[22]

2.4 Properties of ASIC3 channel

The acid-sensing ion channels 3 (ASIC3) is one of seven member of the ASIC channel family (ASIC1a, ASIC1b, ASICb2, ASIC2a, ASIC2b, ASIC3, and ASIC4), which can be activated by extracellular proton and make them pH sensors for cells. The distributions of the ASIC family are different as ASIC1a and ASIC2 are presented both in CNS and PNS; however ASIC1b and ASIC3 are restricted to PNS.[46] Beside from proton sensing, ASIC3 channel is also suggested to be a mechanosensitive channel.[47,48] Similar to TRPV1 and TRPV4, ASIC3 is related to local ATP release after stimulation.[20]

ASIC3 was reported to play a vital role in inducing acid-induced muscle pain created by repeated pH 4 saline injection at gastrocnemius muscle. Jerzy Karczewski et al.[49] reported APETx2, an ASIC3 specific antagonist, reduced mechanical hypersensitivity in rodent acid-induced muscle pain

model created by repeated pH 4 saline injection. They concluded that ASIC3 is the major sensing component when injecting pH 4 saline to muscle. This implies pH4 saline injection is capable of activating ASIC3 in vivo.

2.5 Relationship between acupoint and the mechanosensitive channels : TRPV1, TRPV4, and ASIC3

Recently there are papers implying the involvement of the mechanosensitive TRPV1, TRPV4, and ASIC3 channels during acupuncture. First, as demonstrated by Yamamoto *et al.*[25] that when applying manual acupuncture (MA) or electroacupuncture (EA) to ST36 of rats, both heart rate and blood pressure would transiently attenuate during acupuncture stimulation. However, if the stretch-activated channel blocker gadolinium was given systemically during acupuncture, the attenuation effects by acupuncture were depressed. This strongly suggests that at least a stretch-activated channel is participated during MA. Notably, TRPV1, TRPV4, and ASIC3 are channels that could be non-selectively blocked by gadolinium. Furthermore, Abraham et al.[50] revealed that TRPV1 is more abundantly expressed in subepidermal nerve fibers of BL40 and BL57 acuppoints compared to non-acupoint. Also, TRPV1 in subepidermal nerve fibers of acupoint increased after EA.

2.6 Noxious stimulus-induced analgesia (NSIA)

The noxious stimulus-induced analgesia (NSIA) is a heterosegmental antinociception in which pain sensation is reduced by another pain impulse. This pain-induced analgesia can be generated by hot water (50°C) or subdermal capsaicin injection and can be equivalent in magnitude to that of high dose morphine in jaw-opening reflex.[51] Depending on the site of noxious regulation, mechanism for NSIA could be divided into the spinal pathway and the supraspinal pathway.

For the spinal pathway, when capsaicin is injected into subcutis, TRPV1 activates primary afferent fiber and leads to release of substance P at the laminae I, III and IV of spinal cord. This in turn activates Neurokinin 1 (NK1) receptor of substance P on inhibitory interneuron.[52] Other than substance P, NSIA can also lead to release of glutamate at spinal lamina which also causes activation of inhibitory interneuron by ionotropic glutamate receptors.[53] The activated inhibitory interneuron responses by increases γ-aminobutyric acid (GABA) or glycine release at nociceptive related lamina II. As a result, noxious impulse to the lamina II is negatively regulated.

For the supraspinal pathway, modulation between the spinal lamina and the nucleus accumbens plays a major role. In normal physiological status, tonic activity by spino-supraspinal projection inhibits activity from the nucleus accumbens, which after activation turns on pain modulating Habenula-PAG-NRM-spinal dorsal horn pathway.[54,55] However, when NSIA was introduced by capsaicin, primary afferent stimulated by capsaicin release glutamate at spinal lamina and activate inhibitory interneuron by NMDA receptor, AMPA/kainite receptor and mGluR5 receptor. The activated interneuron then suppresses the inhibitory spino-supraspinal projection by releasing opioid and GABA. As a result, the nucleus accumbens is freed from inhibition and turns on the opioid related pain

modulating Habenula-PAG-NRM-spinal dorsal horn pathway.[56]

It's noteworthy that aside from capsaicin, NSIA can also be activated by agonist of P2X receptor, αβ-methylene-ATP. This was demonstrated by increased frequency of the spontaneous inhibitory postsynaptic currents (sIPSC) from the spinal lamina II after applying αβ-methylene-ATP as when capsaicin was applied.[53]

2.7 Calcium wave propagation (CWP)

It's worth mentioning that the local ATP released is related to intercellular purinergic signaling called calcium wave propagation (CWP).[26,57] Once activated by extracellular ATP via purinergic P2Y receptors (P2Y1 or P2Y2 receptors), the stimulated cells are then processed through intracellular calcium signaling, resulting in ATP release by hemi-channels (e.g., pannexin 1 or connexin 43). ATP released from the cells then stimulates purinergic receptors in the nearby cells in a paracrine manner and causes again both intracellular calcium signaling and ATP release. This chain-like process can continue for a certain distance before termination. The phenomenon is universal and is reported among glia,[26] salivary glands,[27] nephrons,[28] fibroblasts,[29] etc. Recently, CWP during acupuncture was reported at acupoint in both neural and non-neural cell.[31]

It was proposed in keratinocytes[30] and subepithelial fibroblasts[29] of villi that cells respond to mechanical stimulation by local ATP release. Then, by way of CWP, the stimulation signal was conduct to nearby neuron indirectly. This demonstrates an alternative sensing pathway other than direct neural stimulation.

Chapter 3 : Materials and Methods

3.1 Animal

Experiments were carried out on ICR mice (aged 8 to 12 weeks) purchased from BioLASCO Co., Ltd, Taipei, Taiwan. After arrival, 12hr light-dark cycle with sufficient water and food were given. All procedures were approved by the Institute of Animal Care and Use Committee of China Medical University (permit No. 101-116-N) and were in accordance with *Guide for the use of Laboratory Animals* by National Research Council[58] and with the ethical guideline of the International Association for the study of pain.[59] The number of animal used and their suffering were minimized.

3.2 Inflammatory Pain Model and Behavior Test

To generate an inflammatory pain model, mice were anesthetized with 2% isoflurane, and 20 μL CFA (1:1 mixture of saline with complete Freund's adjuvant; Sigma-Aldrich, St. Louis, MO, USA) was subcutaneously injected into the right hind paw.[60] MA and agonist injections were given once on day 3 (D3).

A thermal hyperalgesia test was performed using Hargraves' test IITC analgesiometer (IITC Life Sciences, Woodland Hills, CA, USA). The test was performed on day 0 before CFA injection (D0), on day 3 before intervention (MA or drug injection) (D3 pre), on day 3, approximately 60 min after intervention (D3 post), and on D4, which was 24 h after intervention. To perform the test, a radiant heat source was focused on the right hind paw, and withdrawal latency was determined as the time taken for paw removal. During each tested time point, five repeated tests were conducted, and the average was calculated. To avoid damage to tissues, a resting interval of at least 4–5 min was set between tests, and the maximum

time of heat focus was 20 s. To minimize the effect of isoflurane, 2% isoflurane was given 60 min before each test, for approximately 30 min with or without intervention. The averaged withdrawal latency of every time point was divided by the averaged latency recorded on D0, as the final withdrawal latency ratio, to minimize individual variance among mice. For comparisons between groups, the change of ratio was calculated by simply subtracting the ratio recorded on D3 before intervention from the ratio of selected time points.

3.3 Manual Acupuncture

After anesthesia with 2% isoflurane, MA was performed by inserting a stainless steel acupuncture needle (diameter, 0.16 mm; length, 7.5 mm; Shinlin CO., Ltd, Tianjin, China) into ipsilateral Zusanli acupoint (ST36) of the inflamed limb. The location of ST36 is approximately 4 mm below and 1–2 mm lateral to the midpoint of the knee in mice. An ipsilateral nonacupoint located around the midpoint of the superior edge of the gluteus maximus muscle was selected as the sham. ST36 was selected because of its well-recognized analgesia effect in mouse pain models, and the sham point was used because of the relative scarce acupoints located in the region.[19,61,62] This sham point was also suitable because it is located between two meridians in the region, the urinary bladder and gallbladder meridians, and is a distant from the frequently used acupoint GB40.[62] To ensure an insertion depth of 3 mm, a piece of tape was stuck to the needle, leaving only space for manipulation and a needle tip of 3 mm. During acupuncture, the tape was used as a guide and a twisted was made with the needle turned 360° anticlockwise, then back for one twist at a speed of approximately 1 turn/s. A protocol of two twists every 5 min for duration of 30 min was followed as described by Goldman *et al*.[19] Tests on the needle group were performed by only inserting a needle at ST36, without any twisting. Tests on the sham

group were performed by manipulation as MA at the sham point. The first behavioral test after MA was performed 50–70 min after acupuncture, which represented an average of 60 min after MA.

3.4 Drugs and Injection Method

The TRPV1 agonist capsaicin (0.5%; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 5% ethanol, 5% Tween-20, and 90% saline. The concentration of capsaicin was selected based on the report by Gear *et al.*: a concentration of 0.5% yielded the maximal noxious stimulus-induced analgesia.[51] The TRPV4 agonist GSK1016790A (Sigma-Aldrich, St. Louis, MO, USA) was given at concentrations of 0.02% (almost saturated in the vehicle used), 0.01%, and 0.001%, respectively, in 5% DMSO, 5% Tween-20, and 90% saline. These concentrations were chosen after calculating the ratio of the concentration used and the half-maximal effective concentration (EC50) provided by the drug company to achieve a ratio of GSK1016790A similar to the ratio used for capsaicin injections, because capsaicin was demonstrated to replicate analgesia according to our results. Acidified saline solutions (pH 5, 4, and 3) were used as agonists of ASIC3. They were prepared using 0.01 M 2-[*N*-morpholino] ethanesulfonic acid (MES) dissolved in saline and pH-adjusted with 0.1 M HCl or NaOH. These pH values were selected because Jerzy Karczewski *et al.*[49] reported that APETx2, an ASIC3-specific antagonist, reduced mechanical hypersensitivity in a rodent acid-induced muscle pain model created by repeated injection of pH 4 saline . They concluded that ASIC3 is the major sensing component after injection of pH 4 saline into muscle. A pH 7.4 vehicle control was prepared as an injection fluid without drugs.

After anesthesia with 2% isoflurane, 10 μL of the drug or vehicle were injected 3 mm deep at ST36 or the sham acupoint (located as described in the MA method). GSK1016790A and acidified saline were injected into

ST36 only. The first behavioral test after the injection was performed 50–70 min later, representing an average of approximately 60 min. All animals were grossly normal during behavioral tests.

3.5 Tissue Sampling and Western Blotting

Mice were initially anesthetized with an overdose of choral hydrate and intracardially perfused with saline. Samples of subcutaneous loose connective tissue (ScLCT), epimysium, muscle tissue, and the deep peroneal nerve were collected at ST36. After skin dissection, the ScLCT (with the appearance of a ground-glass-like sheet) overlying ST36 was pulled up lightly using microforceps and retrieved with a microscissor **(Fig. 1B)**. Subsequently, microforceps were used to gently and bluntly dissect the cut edge of the ScLCT to clear the remaining ScLCT. The epimysium, a whitish membrane overlying the anterior tibia muscle, was identified. Because ST36 is located at the medial side of the anterior tibia muscle, thus a vertical incision on the midline of the muscle belly was made and another along the lateral border of the tibia. Subsequently, the upper portion of the epimysium was taken (approximately 20% of the tibia length) **(Fig. 1C)**. It is important to try separating any muscle tissue remaining on the epimysium. Muscle tissue located directly under the sampling field of the epimysium was gathered **(Fig. 1D)**. To dissect the deep peroneal nerve, first the sciatic nerve at the mid-thigh level had to be identified and then dissection was made along the track of the nerve to identify the common and deep peroneal nerves. The upper quarter of the deep peroneal nerve was cut and part of the common peroneal nerve, near the fibula, was taken as a nerve sample **(Fig. 1E)**. A similar sampling method was applied at the sham point **(Fig. 1G and H)**; however, the epimysium and nerve tissue were not retrieved because of technical difficulties.

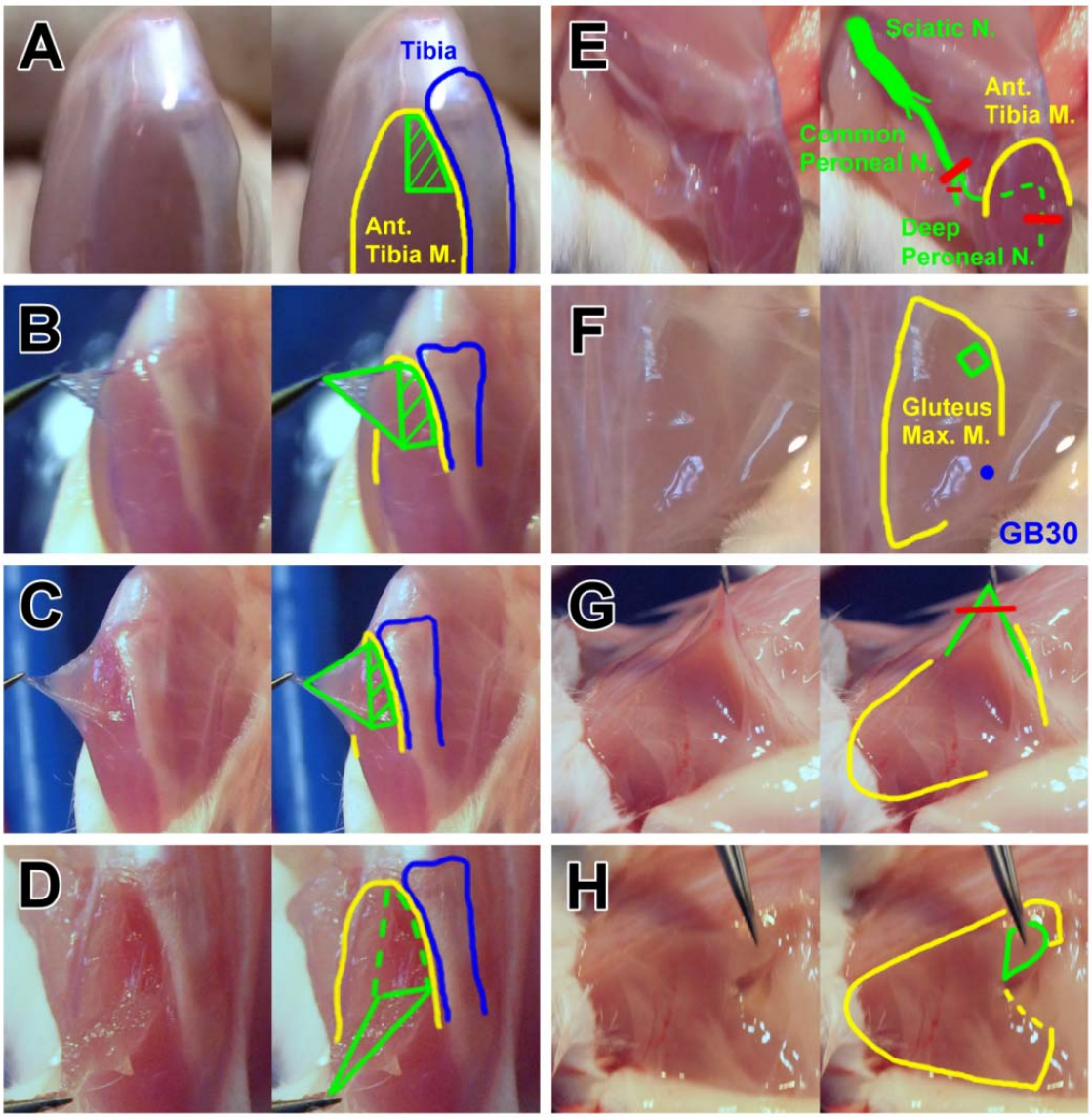

Figure 1. Tissue sampling from ST36 and the sham point

(A) ST36 was located 4 mm below and 1–2 mm lateral to the midpoint of the knee, and **(F)** the sham point was defined at the midpoint of the superior edge of the gluteus maximus muscle. At ST36, **(B)** subcutaneous loose connective tissue (ScLCT), **(C)** epimysium, **(D)** muscle, and **(E)** nerve were obtained (green areas). At the sham point, only **(G)** ScLCT and **(H)** muscle were obtained because of technical issues. Margin of the muscle (yellow), bone (blue), and a frequently used acupoint near the region (GB30).

Sampled proteins were prepared by adding lysis buffer containing 50 mM Tris-HCl pH 7.4, 250 mM NaCl, 1% NP-40, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 0.02% NaN_3, and 1× protease inhibitor cocktail (AMRESCO, Solon, OH, USA) to samples. They were then homogenized using a Bullet Blender homogenizer (Next Advance, NY, USA). The extracted proteins (30 μg/sample, as assessed using the BCA protein assay) were subjected to 8% SDS-Tris glycine gel electrophoresis and transferred to a PVDF membrane. The membrane was blocked with 5% nonfat milk in TBS-T buffer (10 mM Tris pH 7.5, 100 mM NaCl, and 0.1% Tween-20) and incubated with the appropriate antibody overnight at 4°C in TBS-T with 1% bovine serum albumin (BSA). The primary antibodies used were anti-TRPV1 (1:1000), anti-TRPV4 (1:1000), anti-ASIC3 (1:500), and anti-P2Y1 (1:500) from Alomone, Jerusalem, Israel; anti-pannexin 1 (1:125) and anti-connexin 43 (1:500) from Invitrogen, New York, USA; anti-PGP9.5 (1:250) and anti-P2Y2 (1:500) from Abcam, Cambridge, MA, USA; and anti-α-tubulin (1:1000) from Santa Cruz, Dallas, Texas, USA. A peroxidase-conjugated anti-rabbit or anti-mouse (1:10,000) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used as a secondary antibody. The bands were enhanced using a chemiluminescence kit (T-Pro Biotechnology, New Taipei, Taiwan) and visualized with LAS-3000 Fujifilm (Fuji Photo Film Co. Ltd, Tokyo, Japan) or Fusion-SL (Vilber Lourmat, France). The intensities of specific bands were quantified with the NIH ImageJ software (Bethesda, MD, USA). The ratios of proteins were obtained by dividing the intensities of target proteins by the intensity of α-tubulin from the same sample. The calculated ratios were then adjusted by dividing the ratios from the same comparison group to those of the control (muscle or ScLCT from the sham point). Note that the epimysium is a histologically dense connective tissue but shares similar cell types with ScLCT (mostly fibroblasts). The most important difference between the two is cell density; thus, they were placed in the same comparison group after normalization.

3.6 Immunofluorescence

Animals were anesthetized with an overdose of choral hydrate and intracardially perfused with saline. Two cutting sections, located 5 mm above and below ST36, were made vertical to the tibia bone. The samples collected were decalcified in 13% EDTA (pH 7.3) for 3 days and then placed in 30% sucrose overnight and embedded in OCT at −20°C the following day. Frozen sections were cut (30 μm) and placed on glass microslides coated with APS. Subsequently, the sections were postfixed in 4% paraformaldehyde for 3 min and incubated in blocking solution containing 3% BSA, 0.1% Triton X-100, and 0.02% NaN_3 in PBS for 2 h at room temperature. After blocking, sections were incubated with the appropriate primary antibodies in blocking solution at 4°C overnight. Note that sections were incubated in blocking solution without a primary antibody for the negative control. The primary antibodies used were: anti-TRPV1 (1:500), anti-TRPV4 (1:500), and anti-ASIC3 (1:400) from Alomone; and anti-PGP9.5 (1:200) from Abcam. The secondary antibody was a goat anti-rabbit (1:500) antibody (Molecular Probes, Carlsbad, CA, USA). Slides were mounted with cover slips and visualized using a fluorescence microscope (CKX41 with an Olympus U-RFLT50 Power Supply Unit; Olympus, Tokyo, Japan). During microscopic observation, ST36 was defined as described below. First, an imaginary line connecting the tibia and the fibula was set. The point located at the medial one third of the line was identified and ST36 was defined as the projection from that point to the dermomuscular junction of the anterior tibia muscle.

3.7 Statistical Analysis

All statistic data are presented as the mean ± standard error of the mean. Statistical significance was tested using Mann-Whitney Rank Sum Test ($P < 0.05$ was considered statistically significant).

Chapter 4 : Results

4.1 Manual acupuncture had an analgesic effect at ST36 but not at the sham point

Before demonstrating the existence of mechanosensitive channels at ST36, it was important to make sure that ST36 and the sham point defined were truly a functional acupoint and a nonfunctional sham point, respectively. The results demonstrated MA at the defined ST36, but not at the sham point, effectively relieved thermal hyperalgesia in Hargraves' thermal test in a mouse CFA inflammatory pain model **(Fig. 2 A−C)**.

The results showed that 60 min after MA at ST36 on D3, thermal hyperalgesia was significantly reduced compared with that observed before MA because the withdrawal latency ratio increased from 0.71 ± 0.04 to 0.91 ± 0.07 ($P < 0.05$; Mann−Whitney rank sum test) **(Fig. 2A)**. On D4, 24 h after MA, the withdrawal latency ratio in the MA group was 0.96 ± 0.09; this remained significant compared with the ratio observed before MA on D3 ($P < 0.05$). The change in latency ratio among the tested groups on D3 were calculated as follows: the MA group showed a change of 0.20 ± 0.04, the CFA group (without intervention) exhibited a change of 0.00 ± 0.12, the sham group (acupuncture manipulation at the sham point) showed a change of 0.08 ± 0.05, and the needle group (insertion at ST36 without manipulation) showed a change of 0.07 ± 0.03 **(Fig. 2B)**. Significant differences regarding change of ratio were observed only between the MA group and the remaining three groups ($P < 0.05$; Mann−Whitney rank sum test). However, there was no significant difference between the change of ratio among any of the groups on D4 **(Fig. 2C)**. The difference in the results of within-group comparisons and between-group comparisons on D4 was because of a relatively larger variation on D4. This may reflect a variation in the MA

therapeutic time between individual mice. The observation that the analgesic effect was only observed in the MA group implies that acupuncture analgesia can only be induced on acupoints and that manipulation is vital for the effect. Moreover, the locations of the functional acupoint ST36 and the nonfunctional sham point were as defined.

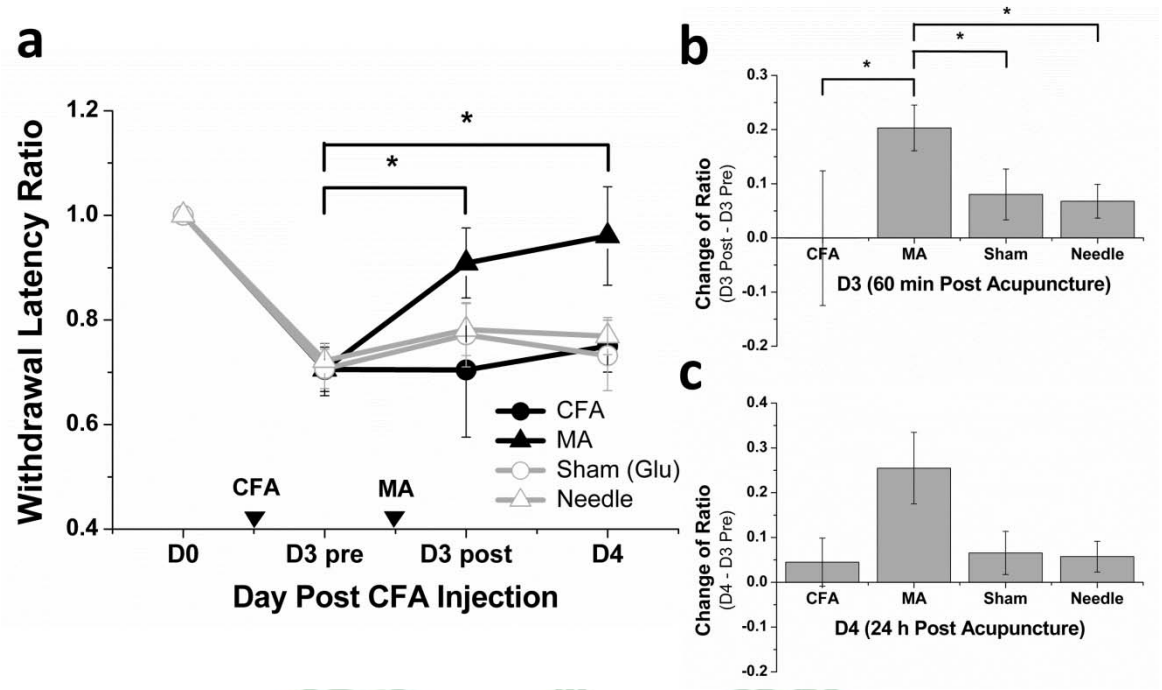

Figure 2. Manual acupuncture (MA) at ST36 yielded an analgesic effect in a mouse inflammatory pain model that was created using complete Freund's adjuvant (CFA)

On day 3 (D3) after CFA injection, MA, sham treatment (Sham), or needling without twisting (Needle) were administered at ST36 or sham point. Hargraves' thermal test was performed before CFA injection (D0), on D3 preintervention (D3 pre), on D3 postintervention (D3 post, 60 min after intervention), and on D4 (24 h after intervention). **(A)** MA at ST36 yielded an analgesic effect in within-group tests on D3 post ($P < 0.05$) and on D4 ($P < 0.05$). **(B)** Between-group tests showed that MA yielded significant differences compared with CFA ($P < 0.05$), Sham ($P < 0.05$), and Needle ($P < 0.05$) groups on D3 post. **(C)** No significant differences were observed on D4 in between-group tests (*$P < 0.05$, Mann–Whitney rank sum test, $n = 9$–14). Data are means ± S.E.M.

4.2 Mechanosensitive channels were abundantly expressed at ST36

After assuring that the locations of the functional ST36 and the nonfunctional sham point were as defined, samples from the two points were gathered to determine if there were differences in the expression of the mechanosensitive channels TRPV1, TRPV4, and ASIC3 using Western blotting. Neural tissue (Ner, deep peroneal nerve), subcutaneous loose connective tissue (ScLCT), epimysium (Epi), and muscle (Mus) were obtained from ST36. Because of technical difficulties, only subcutaneous loose connective tissue and muscle were obtained from the gluteus sham point. Notably, although epimysium is a dense connective tissue, the tissue share similar cellular components with the ScLCT (mostly fibroblasts), albeit with different cell densities. Therefore, after normalization, they were still placed in the same comparison group.

The results of this experiment demonstrated that all three channels were positively expressed in neural tissue. TRPV1 was abundantly expressed at ST36 muscle (1.89 ± 0.32-fold over the sham, $P < 0.05$; Mann–Whitney rank sum test) compared with the sham. TRPV1 was abundantly expressed at ST36 epimysium compared with sham ScLCT (2.23 ± 0.47-fold, $P < 0.05$) and ST36 ScLCT (2.23 ± 0.47 vs. 1.17 ± 0.07, $P < 0.05$) **(Fig. 3A)**. TRPV4 was expressed in muscle, but no difference was found between ST36 muscle and the sham (1.10 ± 0.18-fold vs. the sham). A significant difference was observed between ST36 ScLCT and ST36 Epi (1.44 ± 0.20 vs. 0.91 ± 0.06, $P < 0.01$) and between ST36 ScLCT and sham ScLCT (1.44 ± 0.20-fold, $P < 0.01$) **(Fig. 3B)**. ASIC3 was more abundant in ST36 muscle than in the sham (1.21 ± 0.07-fold, $P < 0.05$) and was predominantly expressed in ST36 epimysium compared with ST36 ScLCT (1.45 ± 0.12 vs. 1.14 ± 0.12, $P < 0.05$) and sham ScLCT (1.45 ± 0.12-fold, $P < 0.01$) **(Fig. 3C)**.

To verify if the expression patterns observed could be attributed to differences in neural distribution or differences in the expressed levels of

channels, the expression pattern of the pan-neuronal maker PGP 9.5 was tested. PGP 9.5 was expressed in neural, muscle, epimysium, and ScLCT tissues. This demonstrated neural innervation in the anatomical layers. However, there was no difference in expression in muscle or connective tissue comparing ST36 with the sham point **(Fig. 3D)**. This indicated that there was no difference in neural distribution between ST36 and the sham point. The abundance in TRPV1, TRPV4, and ASIC3 shown at ST36 was the result of an increased number of channels expressed in the anatomical layers.

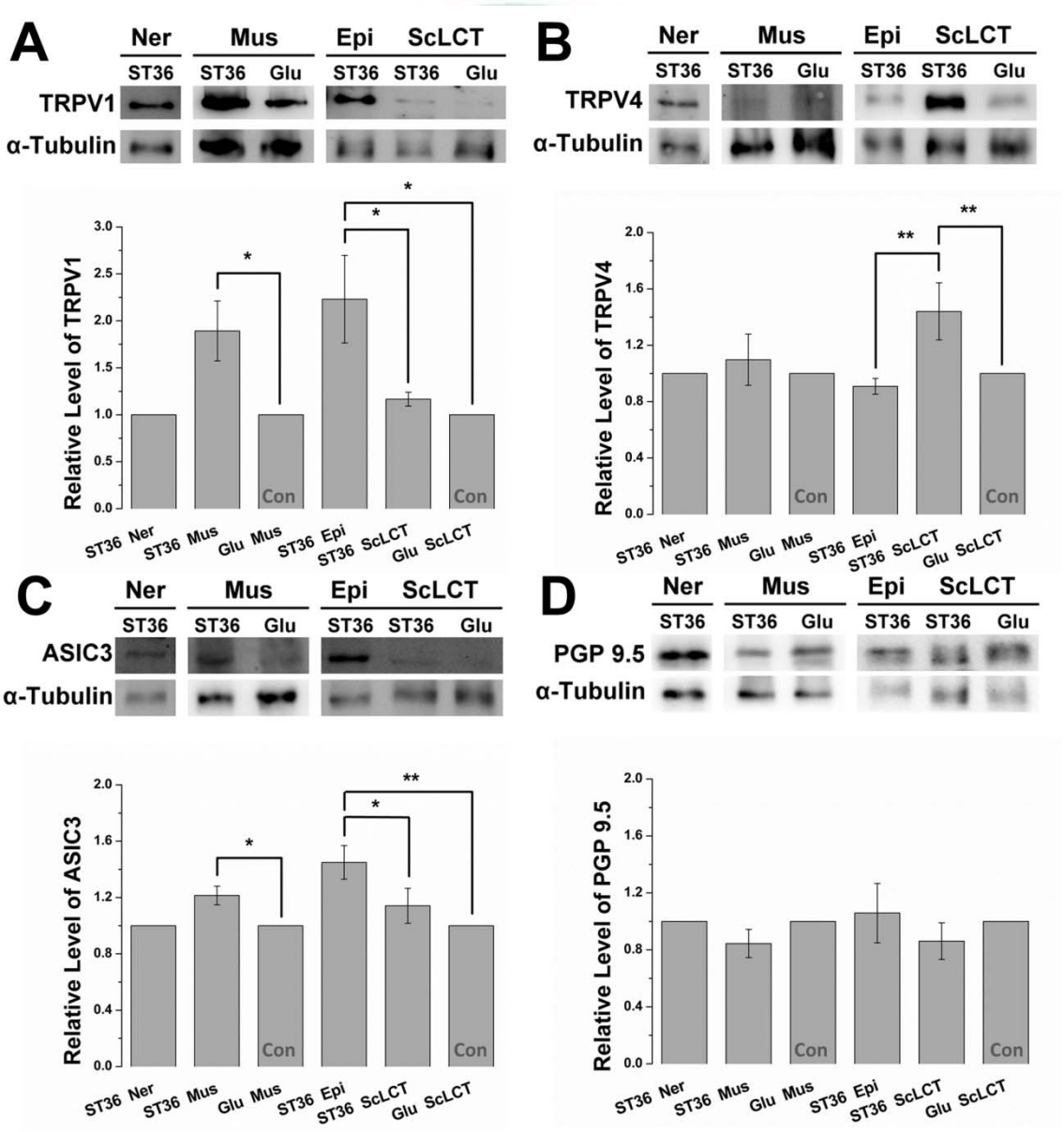

Figure 3. Western blotting showed that the mechanosensitive channels TRPV1, TRPV4, and ASIC3 were abundantly expressed in different anatomical layers of ST36

Tissues were sampled from nerve (Ner), muscle (Mus), epimysium (Epi), and subcutaneous loose connective tissue (ScLCT) of ST36. Only muscle and ScLCT were obtained from the sham point because of technical issues and were both used as control (Con) in separate comparison groups. **(A, B, C, and D)** TRPV1, TRPV4, ASIC3, and PGP 9.5 (a pan-neural marker) were expressed in nerve, muscle, and connective tissue. **(A)** TRPV1 was abundantly expressed at ST36 muscle ($P < 0.05$) compared with sham, and ST36 epimysium exhibited higher expression compared with ST36 ScLCT ($P < 0.05$) and sham ScLCT ($P < 0.05$). **(B)** TRPV4 was abundantly expressed at ST36 ScLCT compared with ST36 epimysium ($P < 0.01$) and sham ScLCT ($P < 0.01$). **(C)** ASIC 3 exhibited higher expression at ST36 muscle compared with the sham ($P < 0.05$), and at ST36 epimysium compared with ST36 ScLCT ($P < 0.05$) and sham ScLCT ($P < 0.01$). **(D)** PGP 9.5 was not significantly expressed at ST36 or the sham point, which indicated an absence of differences in nerve distribution in these two points. The relative level of target proteins was calculated by dividing the ratio of targeted proteins (targeted protein/α-tubulin) by the ratio of control in the comparison group. ST36 epimysium, ST36 ScLCT, and sham ScLCT were placed in the same comparison group because their cellular components were similar (mostly fibroblasts), even though their cell densities were different (*$P < 0.05$, **$P < 0.01$; Mann–Whitney rank sum test; TRPV1, $n = 6$; TRPV4, $n = 7$; ASIC3, $n = 7$–10; PGP 9.5, $n = 9$). Data are means ± S.E.M.

4.3 Mechanosensitive channels were expressed in neural and non-neural cells

The experiments described above showed that TRPV1, TRPV4, and ASIC3 were abundantly expressed in the anatomical layers of ST36. Nonetheless, the results of Western blotting did not reveal the histological expression of channels. Therefore, immunofluorescence at ST36 was performed **(Fig. 4A–H)**. First, ST36 location was microscopically defined. The microscopic section showed that all three channels were expressed in subcutaneous nerve fibers (arrow) **(Fig. 4B, D, and F)**. They were also expressed in muscle, particularly in the cell membrane (higher expression at the margin of muscle fibers). This correlates with their role as membrane channels. Higher fluorescence was observed in muscle fibers labeled for TRPV1. In contrast, TRPV4 showed a relatively lower expression. This difference in expression is consistent with the findings of Western blotting because TRPV1 exhibited the highest relative level of expression. Interestingly, only for TRPV1, the microscopic slide showed a very thin layer at the margin of the muscle beneath the epimysium which exhibited even higher expression **(Fig. 4A)**. This interested us because acupuncture sensation (de-qi) is stronger just after the needle tip enters the perimuscular fascia (epimysium).[63]

All three channels showed positive expression in the cells of the epimysium (between green lines) **(Fig. 4B, D, and F)** and subcutis (arrowhead). In accordance with the results of Western blotting, expression in subcutaneous cells seemed relatively evident for TRPV4. It is logically to consider that the positive cells observed in the epimysium and subcutis are mainly fibroblasts, taking into account that fibroblasts are the principal cell in connective tissue[64] and that TRPV1[65], TRPV4,[66,67] and ASIC3[68,69] are reported to express in fibroblast. However, these positive cells in the epimysium and subcutis could be cells other than fibroblasts, such as mast

cells. Immunofluorescence experiments showed that TRPV1, TRPV4, and ASIC3 were expressed in neural and non-neural cells (muscle cells and maybe fibroblasts).

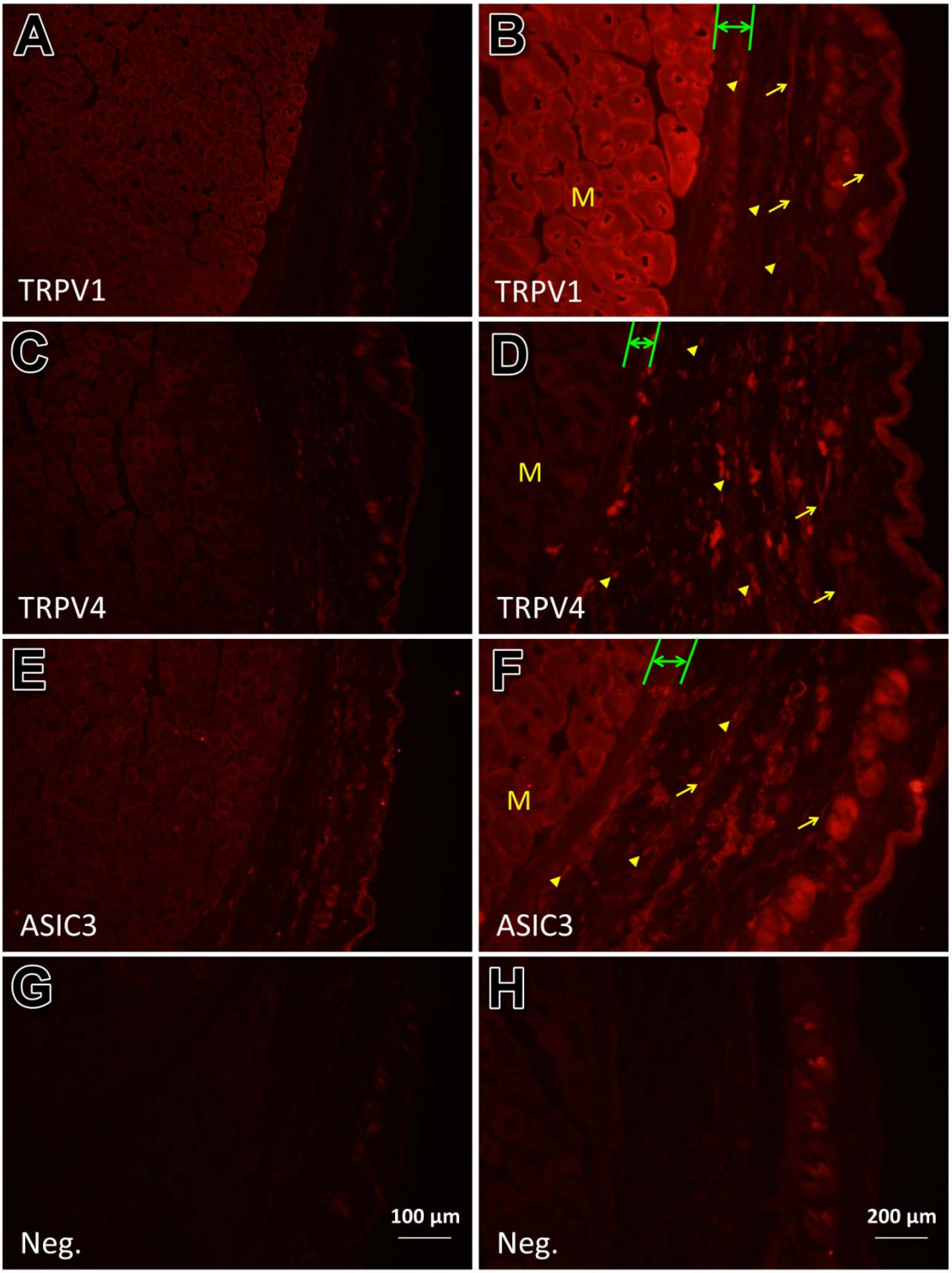

Figure 4. TRPV1, TRPV4, and ASIC3 were expressed in varying type of cell under immunofluorscence

TRPV1 **(A and B)**, TRPV4 **(C and D)**, and ASIC3 **(E and F)** were primarily expressed in muscle fibers (M), nerve (arrow), and subcutaneous cells (arrowhead) (as assessed using immunofluorescence). Immunofluorescence results correlated with the patterns observed in Western blots. TRPV1 and ASIC3 showed evident expression in muscle and epimysium (between green lines), and TRPV4 was evident in subcutaneous cells. Note that stronger fluorescence was observed at the cell margin in muscles, which is in accordance with their role as membrane channels. **(A)** In particular, TRPV1 was expressed more strongly at a very thin layer located beneath the epimysium. (**A, C, E, and G)** Captured under the same microscopic magnification, and **(B, D, F, and H)** captured under a different magnification. Scale bars are as shown. **G and H,** negative controls (no primary antibody).

4.4 Injection of the TRPV1 agonist capsaicin into ST36 replicated the acupuncture-like analgesic effect

Next, it is important to determine whether the activation of these channels would produce an acupuncture-like analgesic effect. This was achieved by injecting 10 μL of agonist into ST36 and testing in the mouse CFA inflammatory pain model.

In the case of TRPV1, 0.5% capsaicin was injected that resulted in an analgesic effect similar to that of MA **(Fig. 5A–C)**. On D3, the withdrawal latency ratio increased from 0.70 ± 0.05 before injection to 1.01 ± 0.08 after injection ($P < 0.01$; Mann–Whitney rank sum test) **(Fig. 5A)**. The antinociceptive effect of capsaicin injection persisted on D4, with a ratio of 0.87 ± 0.04 ($P < 0.05$ compared with the ratio recorded on D3 pre). The comparison between groups on D3 revealed that change of ratio was significantly different between the capsaicin and sham groups (injection of 10 μL 0.5% capsaicin into the sham point) (0.31 ± 0.09 vs. −0.06 ± 0.07, $P < 0.01$) **(Fig. 5B)**. Similarly, the comparison of the capsaicin and vehicle groups showed significant differences (0.31 ± 0.09 vs. 0.03 ± 0.05, $P < 0.05$). However, no significant difference in change of ratio was found between groups on D4 **(Fig. 5C)**.

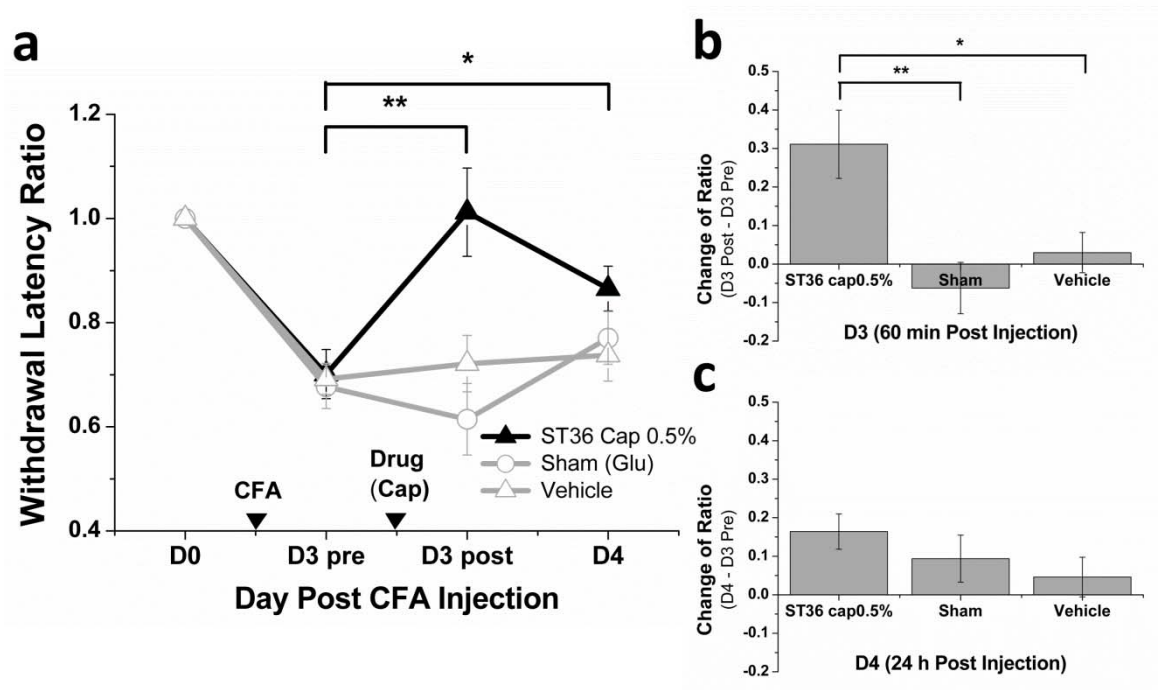

Figure 5. TRPV1 agonist (capsaicin) injection at ST36 replicated the acupuncture-like analgesic effect

The behavioral test design was similar to that of MA, with the exception that capsaicin was injected at a concentration of 0.5% and a volume of 10 μL into ST36 or sham point. **(A)** In within-group tests, capsaicin injections into ST36 showed significant effect on D3 post ($P < 0.01$) and on D4 ($P < 0.05$). **(B)** In between-group tests, capsaicin injections at ST36 yielded significant differences compared with injections at the sham point ($P < 0.01$) and vehicle injections at ST36 ($P < 0.05$) on D3 post. **(C)** There were no significant differences in between-group tests on D4. Within-group and between-group tests were designed similar to the MA behavioral test (*$P < 0.05$, **$P < 0.01$; Mann–Whitney rank sum test, n = 11–13). Data are means ± S.E.M.

For TRPV4, injection of the agonist, GSK1016790A, did not induce an analgesic effect **(Fig. 6A–C)**. Mice were grouped into the vehicle group and groups that received 0.001%, 0.01%, and 0.02% GSK1016790A. There were no significant differences in the withdrawal latency ratio among groups on D3 and D4 **(Fig. 6A)**. The change of ratio in between-group tests were not significant on D3 **(Fig. 6B)** and D4 **(Fig. 6C)**.

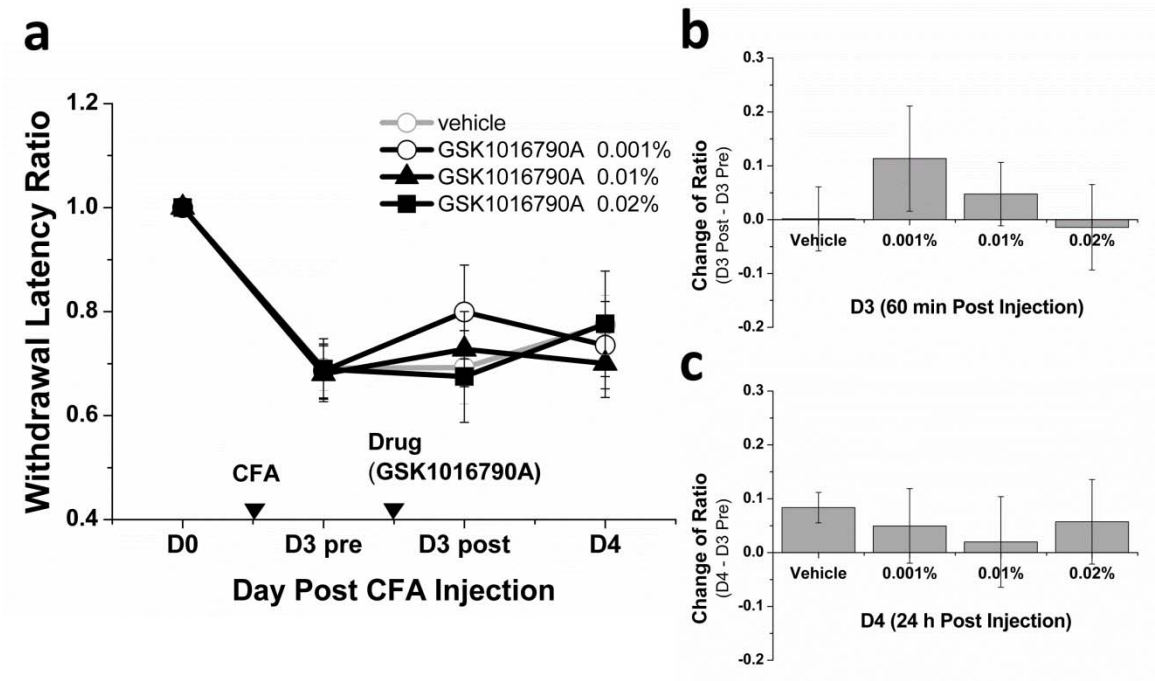

Figure 6. TRPV4 agonist (GSK1016790A) injection at ST36 yielded no analgesic effect

The behavioral test design was similar to that of MA; one difference was that GSK1016790A was injected at a different concentration and a volume of 10 μL into ST36. **(A)** Injections of different concentrations of GSK1016790A did not yield significant differences in within-group tests on D3 post and on D4. **(B and C)** No significant differences were found in between-group tests on D3 post and on D4. Within-group and between-group tests were designed similar to the MA behavioral test (Mann–Whitney rank sum test; *n* = 8–9). Data are means ± S.E.M.

Regarding ASIC3, acidified saline was used as a nonselective agonist and was injected into ST36 at pH 5, 4, and 3. No obvious analgesic effect was observed **(Fig. 7A–C)**. On D3 and D4, no significant differences were found in within-group comparisons **(Fig. 7A)**. Moreover, on D3 post **(Fig. 7B)** and D4 **(Fig. 7C)**, the change of ratio in between-group tests were not significantly different between pH 7.4 normal saline injection and acidified saline injections.

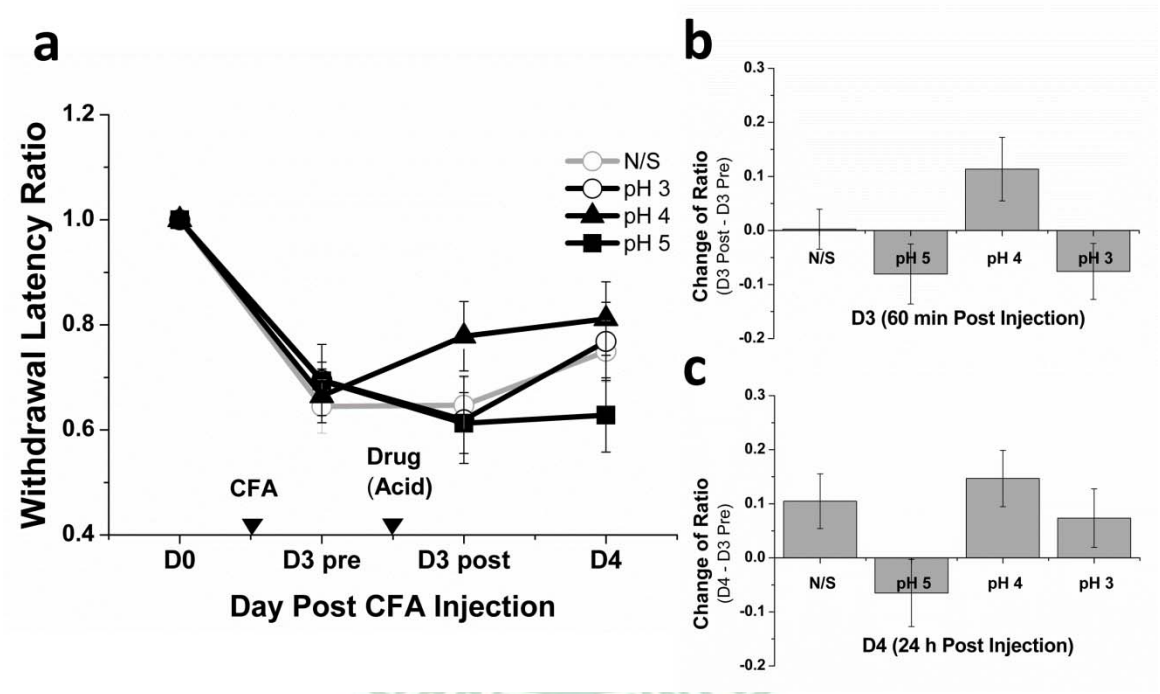

Figure 7. ASIC3 agonist (acidified saline) injection at ST36 yielded no analgesic effect

The behavioral test design was similar to that of MA; one difference was that acidified saline was injected at different pH values in a volume of 10 μL into ST36 only. **(A)** In within-group tests, injections of acidified saline at different pH values did not yield significant differences on D3 post and on D4. **(B and C)** No significant difference was observed between pH 7.4 normal saline (N/S) injection and acidified saline injection on D3 post and on D4. Within-group tests and between-group tests were designed similar to the MA behavioral test (Mann–Whitney rank sum test, n = 9–15). Data are means ± S.E.M.

As only capsaicin produced an analgesic effect similar to that of MA, the change of ratio between MA and 0.5% capsaicin injection were compared. No significant difference was observed between the two treatments on D3 and D4, indicating that 0.5% capsaicin injection at ST36 was as effective as MA. These results suggest that the mechanosensitive TRPV1 is functional at ST36.

4.5 Components of CWP were abundantly expressed at ST36

The questions remain as to why TRPV1 is abundantly expressed in non-neural cells and whether its expression in non-neural cells is related to acupuncture signaling to neurons. Furuya *et al.* stated that after mechanical stimulation of subepithelial fibroblasts of villi by food and water, these cells release ATP to the surrounding medium.[29] This elicits a CWP that activates neuronal terminals. Because CWP was reported during acupuncture in non-neural cells,[31] it is logically then to speculate that non-neural cells at acupoints receive this mechanical signal via TRPV1 and subsequently release ATP after stimulation by acupuncture, as reported in other tissues.[20,21] ATP released by non-neural cells then stimulates nearby neurons via CWP. Although prove of this via calcium imaging was not provided, this possibility was demonstrated by showing that the CWP components (pannexin 1, connexin 43, P2Y1, and P2Y2) and TRPV1 were coexpressed in the same anatomical layers in Western blotting. Moreover, these components were abundantly expressed in ST36.

The results showed that pannexin 1, connexin 43, P2Y1, and P2Y2 were expressed in nerve, muscle, and connective tissue. Furthermore, pannexin 1 was abundantly expressed in ST36 epimysium compared with sham ScLCT (1.47 ± 0.27-fold, $P < 0.05$) and ST36 ScLCT (1.47 ± 0.27 vs. 0.95 ± 0.10, $P < 0.05$, Mann–Whitney rank sum test) **(Fig. 8A)**. Similarly, connexin 43 was abundantly expressed at ST36 epimysium compared with sham ScLCT (1.33

± 0.07-fold, $P < 0.01$) and ST36 ScLCT (1.33 ± 0.07 vs. 1.09 ± 0.05, $P < 0.05$) **(Fig. 8B)**. ST36 muscle exhibited abundant P2Y1 expression compared with the sham (1.50 ± 0.20-fold, $P < 0.05$). Moreover, abundant P2Y1 expression was found at ST36 ScLCT compared with ST36 epimysium (1.76 ± 0.15 vs. 0.74 ± 0.13, $P < 0.01$) and sham ScLCT (1.76 ± 0.15-fold, $P < 0.01$) **(Fig. 8C)**. Abundant P2Y2 expression was observed at ST36 epimysium compared with ST36 ScLCT (1.92 ± 0.31 vs. 1.30 ± 0.25, $P < 0.05$) and sham ScLCT (1.92 ± 0.31-fold, $P < 0.01$) **(Fig. 8D)**.

The occurrence of CWP requires the expression of either the pannexin 1 or connexin 43 hemichannels in an anatomical layer for ATP release, and expression of the P2Y1 or P2Y2 purinergic receptors for ATP signaling. After cross-matching the expression patterns of the components, only muscle and epimysium other from nerve expressed both hemichannels and purinergic receptors. These two layers were also the site at which TRPV1 was abundantly expressed. This coexpression made it spatially possible for TRPV1 to pass on the ATP signal and trigger CWP to neurons.

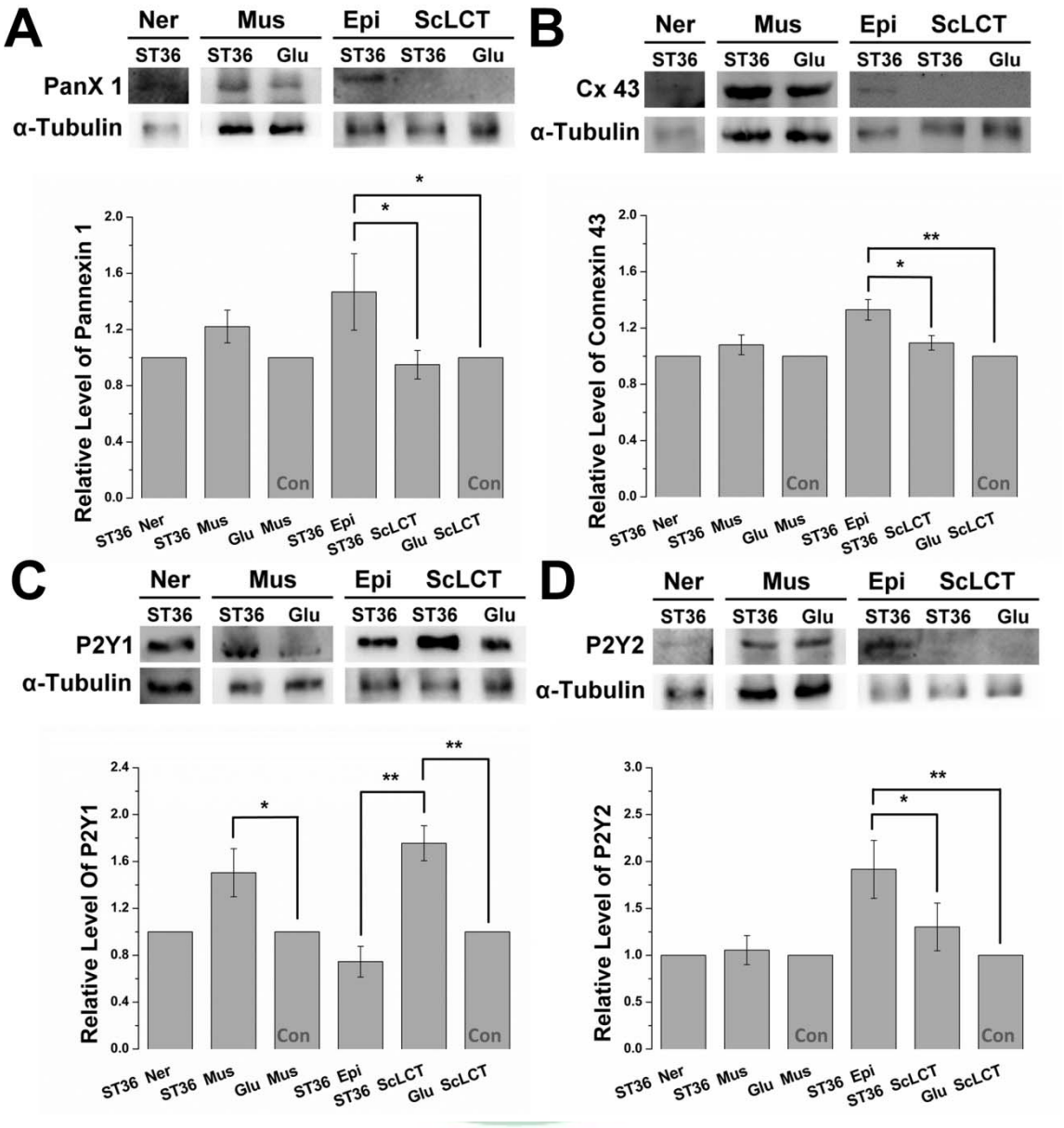

Figure 8. Components for calcium wave propagation (CWP) were expressed abundantly in different anatomical layers of ST36

Components tested were the ATP-releasing hemichannels pannexin 1 (PanX 1) and connexin 43 (Cx 43) and the purinergic receptors P2Y1 and P2Y2. Tissues from nerve (Ner), muscle (Mus), epimysium (Epi), and subcutaneous loose connective tissue (ScLCT) of ST36 or sham were collected in a manner similar to that described for mechanosensitive channel western blotting. **(A, B, C, and D)** Pannexin 1, connexin 43, P2Y1, and P2Y2 were expressed in nerve and muscle. **(A)** Pannexin 1 was more abundantly expressed at ST36 epimysium compared with ST36 ScLCT ($P < 0.05$) and sham ScLCT ($P < 0.05$). **(B)** Connexin 43 was more abundantly expressed at ST36 epimysium compared with ST36 ScLCT ($P < 0.05$) and sham ScLCT ($P <$

0.01). **(C)** P2Y1 was more abundantly expressed in ST36 muscle compared with the sham ($P < 0.05$) and in ST36 ScLCT compared with ST36 epimysium ($P < 0.01$) and sham ScLCT ($P < 0.01$.) **(D)** P2Y2 was more abundantly expressed at ST36 epimysium compared with ST36 ScLCT ($P < 0.05$) and sham ScLCT ($P < 0.01$). All four components were expressed in muscle and the epimysium of ST36. These two layers also abundantly expressed TRPV1. The relative levels of target proteins were calculated in a manner similar to that used for Western blotting of mechanosensitive channels (*$P < 0.05$, **$P < 0.01$; Mann–Whitney rank sum test; pannexin 1, $n = 8–12$; connexin 43, $n = 6$; P2Y1, $n = 5–9$; P2Y2, $n = 7$). Data are means ± S.E.M.

Chapter 5 : Discussion

5.1 Brief Summary of Experiments

After localizing functional ST36, the results showed that TRPV1, TRPV4, and ASIC3 were abundantly expressed in different anatomical layers of this acupoint. Furthermore, the histological results revealed that, not only in nerve fibers, the channels were expressed in skeletal muscle cells and possibly expressed in fibroblasts. The injection of agonists of channels into ST36 showed that only capsaicin replicated acupuncture analgesia. TRPV1 expression in nerves is understandable. Conversely, the abundant expression of TRPV1 in non-neural tissues at ST36 was tried to be explained. After TRPV1 activation, ATP may be released by non-neural cells and trigger CWP, which indirectly conveys signals to nearby nerve terminals. This possibility was confirmed by showing abundant expression of the components of CWP in the anatomical layers of ST36 that also abundantly expressed TRPV1.

5.2 Discussion on anatomical expression of TRPV1, TRPV4, and ASIC3 channels at ST36

The results of immunofluorescence showed that all three channels were expressed in muscle fibers. This was in accordance with previous findings that TRPV1,[70,71] TRPV4,[66,72] and ASIC3[73] are expressed in skeletal muscle. Higher expression of TRPV1 at the muscle margin was accidentally discovered. This is rather interesting because the acupuncture sensation (de-qi) is stronger just after needle-tip insertion into perimuscular fascia (epimysium).[63] However, this warrants confirmation. The microscopic slides also revealed that these channels were expressed in cells of the epimysium and subcutis. TRPV1,[65] TRPV4,[66,67] and ASIC3[68,69] are expressed in

fibroblasts that are the primary cells in connective tissue; thus, the positive cells are suggested to be fibroblasts. If so, mechanosensitive channels may participate in the connective tissue theory proposed by Langevin *et al.*[74-76]

5.3 TRPV1 agonist, capsaicin, induced analgesia might not be related to conduction analgesia

An acupuncture-like analgesic effect of capsaicin injection at ST36 was demonstrated. This is also true in clinical settings, as others reported the acupuncture-like effect of the topical application of capsaicin on acupoints.[77,78] Capsaicin can cause conduction analgesia (or desensitization).[38,39] However, this happens only when capsaicin is delivered to the innervating nerve and functions as lidocaine as conduction blocker. Nonetheless, the deep peroneal nerve innervates ST36. In contrast, the tibia nerve (partiallly the sural and saphenous nerves) innervate the inflamed and tested paws. Anatomically, conduction analgesia on the deep peroneal nerve would have little effect on nerves that innervate the hind paw. Moreover, the deep peroneal nerve is separated from the tibia nerve by an interosseous membrane. Leakage was unlikely because the injection depth was insufficient to penetrate the membrane and the injected volume was minimized to 10 μL. It is more logical to assume that analgesia was caused by a mechanism other than conduction analgesia.

5.4 TRPV1 agonist, capsaicin, induced analgesia might be related to noxious stimulus-induced analgesia

Capsaicin has been used in other experiments to study noxious stimulus-induced analgesia (NSIA).[52,56] Capsaicin stimulation releases glutamate and substance P at the spinal dorsal horn, activating NMDA, AMPA/kainate, mGluR5, and NK1 receptors on inhibitory interneurons and releases GABA or glycine to provide an analgesic effect. In addition to the spinal cord, supraspinal activation of the nucleus accumbens was reported, which turns on the opioid-related descending inhibitory pathway and induces heterosegmental antinociception.[51,54,79] The NSIA mechanism greatly overlaps the proposed acupuncture analgesia mechanism[14-17] and both involve similar spinal and supraspinal regulation. This may explain why capsaicin injection at ST36 replicates MA analgesia. Capsaicin was also injected into the sham point, but with no analgesic effect. There are several logical reasons for this: a nerve trunk (deep peroneal nerve) is closer to ST36 and may result in greater stimulation; nerves supplying acupoints are enriched for TRPV1;[50] the non-neural layers expressed more TRPV1. Higher TRPV1 expression may lead to more ATP release in ST36,[19] suggesting greater purinergic P2X receptor (P2X3 or P2X2) activation that also contributes to NSIA.[53,80] Therefore, it seemed that capsaicin injection at ST36 induces greater TRPV1 and P2X receptor activation and results in better NSIA than that at the sham point.

Our group previously reported that TRPV1, TRPV4, and ASIC3 are upregulated in DRGs after inflammatory pain but attenuated after electroacupuncture.[5,6] Balance recovery is emphasized in traditional Chinese medicine, having hyper-activated to down-regulate and hypo-activated to up-regulate. TRPV1 upregulation observed during pain may serve to enhance NSIA and restore balance.

5.5 Acidified saline used was insufficient to induce the same extent of TRPV1 activation as capsaicin

TRPV1 is a polymodal receptor because it can be activated by various types of stimulation including capsaicin, heat (>43°C), low pH, voltage, and endogenous lipids.[36] As acids can also activate TRPV1, it is worth asking why the acidified saline injection did not activate TRPV1 and provide analgesic effects, similar to capsaicin. Literatures regarding EC50 of capsaicin and acid for TRPV1 were reviewed. Under in vitro conditions at 37°C, EC50 for capsaicin at pH 7.4 is 640 nM and EC50 for acid stimulation is pH 5.35.[81] The ratio for capsaicin used over capsaicin's EC50 is approximately 2.6×10^4, whereas the ratio for acidified saline used over its EC50 is much smaller ($2.2–2.2\times10^2$). This explains why even acidified saline at pH 3 was not sufficient to cause NSIA by TRPV1.

5.6 Possible reasons why TRPV4 and ASIC3 agonist did not replicate analgesic effect

One may ask why TRPV4 and ASIC3 injections did not yield analgesic effects similar to TRPV1. This could be because of the differences in permeability to calcium that acts as modulating messenger. ASIC3 is permeable to sodium, but not calcium, in physiological conditions. TRPV4, although permeable to both cation, is less permeable to calcium than to TRPV1.[82] This may also be because of differential expression of channels on nerve fibers. TRPV1 is mostly expressed in small fibers as C-fibers and Aδ-fibers, whereas TRPV4 and ASIC3 are not restricted to them.[6,45,48] As different fiber types terminate in various spinal lamina, it is reasonable to think that a different effect was introduced. Or, as state in gate control theory of pain that impulse from small fibers (NSIA related C-fibers) is inhibited by impulse from large fibers as A β-fibers.

5.7 Possible involvement of non-neural cell sensing pathway by calcium wave propagation (CWP)

The question remains as to how TRPV1 activation is connected to nerve stimulation. This seems obvious if TRPV1 activation occurs after direct puncturing of the membrane of nerve branches, which generates an action potential. However, in clinical practice, direct puncturing of nerve branches is avoided to prevent potential nerve injury. Therefore, it is more likely that nerve stimulation occurs indirectly. According to Langevin *et al.*, acupuncture causes local tissue displacement.[74-76] It is conceivable that local traction by displacement transfers physical forces to nerves and activates TRPV1 to generate an action potential. Alternatively, TRPV1 expression in nerves results in ATP release,[20,21] which stimulates self-purinergic receptors in an autocrine manner **(Fig. 9A)**. ATP may be released by muscle fibers or fibroblasts. Released ATP then conveys the signal to nerves by CWP, as demonstrated by Furuya *et al.* in villi mechanotransduction and by Koizumi *et al.* in keratinocyte mechanotransduction[29] **(Fig. 9B)**. The involvement of CWP is highly likely because the occurrence of CWP in non-neural cells during acupuncture has been reported.[31] Furthermore, as in the results of experiments, TRPV1 and the CWP components were all expressed in muscle and epimysium, which increases the likelihood that CWP carried on the signaling after TRPV1-related ATP release. CWP participation during acupuncture may explain why acupuncture meridians (or channels) did not fully match the anatomy of nerve innervation. CWP may bridge the gap between the two. Moreover, ATP responding P2X receptors also participate in NSIA;[53,80] thus, CWP may result in increased P2X receptor recruitment and NSIA amplification.

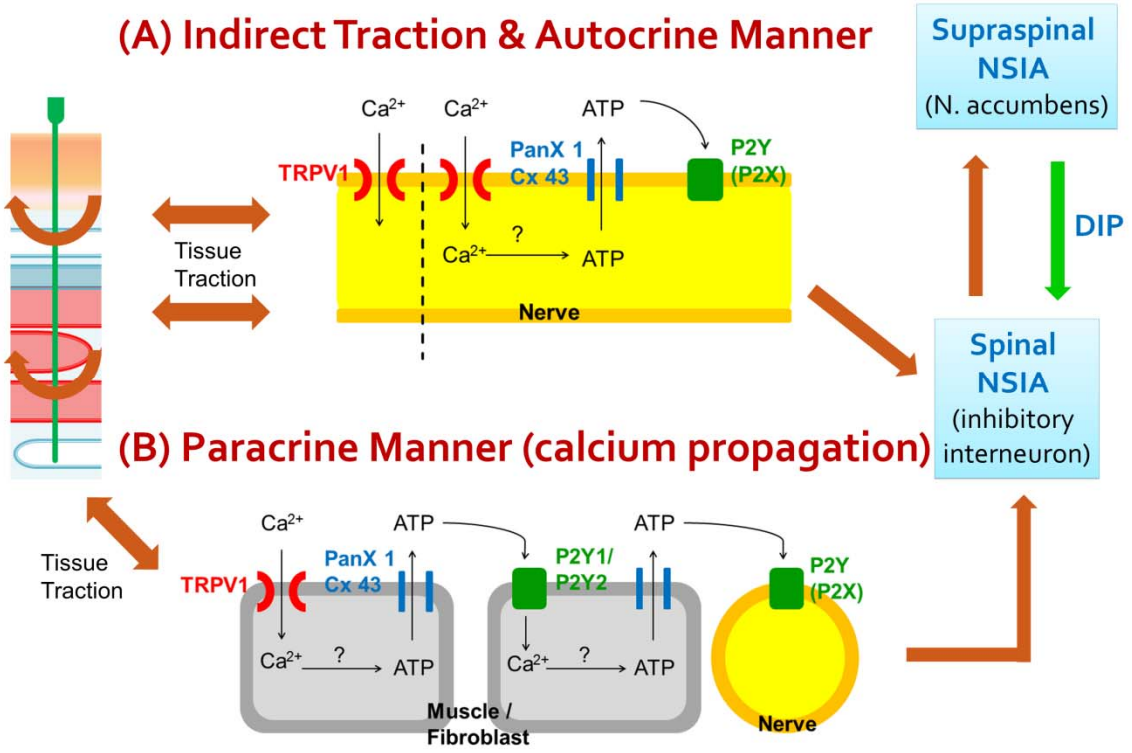

Figure 9. Schematic diagram of the proposed interface between physical stimulation by acupuncture and biological signaling to neurons

Manual acupuncture at acupoints causes tissue traction during manipulation and results in the activation of mechanosensitive TRPV1 on the membrane of cells (neurons, muscle fibers, and fibroblasts). This leads to two parallel sensing pathways: the neural and the non-neural cell initiated sensing pathways. **(A)** In the former, TRPV1 on nearby nerves is stimulated after traction, which generates an action potential after channel opening. It is also possible that increased intracellular calcium leads to ATP release by hemichannels to the extracellular matrix (ECM) after TRPV1 stimulation. The released ATP acts in an autocrine manner and results in self-stimulation (neuron) by purinergic receptors (P2Y or P2X). **(B)** In the latter, TRPV1 on muscle fibers or fibroblasts is activated and increases calcium influx leading to ATP release to the ECM via an unknown signaling pathway. The released ATP then activates purinergic receptors on nearby cells (another muscle fiber or fibroblast). This increases intracellular calcium again and another ATP is released to the ECM. The chain-like paracrine process of ATP release and calcium signaling is named calcium wave propagation (CWP). As in other circumstances, non-neural cells can pass on information to neurons via CWP after traveling for a certain distance. The occurrence of antinociceptive regulation requires that these pathways lead to noxious stimulus-induced analgesia (NSIA), either in the spine (by

inhibitory interneurons) or supraspinally [by the nucleus accumbens and descending inhibitory pathway (DIP)]. This proposed mechanism is supported by previous reports: ATP release during acupuncture (Goldman *et al.*[19] and Takano *et al.*[18]); CWP during acupuncture in non-neural cells (Li *et al.*[31]); signaling from non-neural cells to neurons via CWP (Furuya *et al.*[29] and Koizumi *et al.*[30]); and numerous reports on CWP and NSIA. Abbreviations: pannexin 1 (PanX 1); connexin 43 (Cx 43); noxious stimulus-induced analgesia (NSIA); descending inhibitory pathway (DIP).

5.8 Limitations of the experiments

There were several limitations to the experiments in this thesis. A nonselective acidified saline injection was used to activate ASIC3 because of limitations on the acquisition of commercial ASIC3-selective agonists. The pH value was selected considering that ASIC3 is majorly involved in pH 4 saline-induced chronic muscle pain.[49] The role of ASIC3 during acupuncture would have been better explained if an ASIC3 selective agonist was available. Moreover, directly block the MA analgesic effect with antagonists of mechanosensitive channels was not performed. Most antagonists are already pain relieving when systemically administered,[49,83-85,86] and local injections of antagonists prior to acupuncture may be too damaging for the acupoint. Nonetheless, replicating the acupuncture-like analgesic effect by injecting capsaicin into acupoint ST36 may provide knowledge on the functional role of TRPV1 at acupoints.

5.9 Future Works

Even though systemic or local administration of TRPV1 antagonist have some technical issues to address, experiments designed to demonstrate attenuation of acupuncture effects after TRPV1 blockage is still vital. The following are experiment designs which may potentially solve the issue. From previous experiences, injection at ST36 might affect efficiency of acupuncture if MA is given shortly after injection. This may due to possible injury of ST36 from injection. However, in the case of siRNA injection, the time point of injection can be days before MA. This gives sufficient time for tissue recovery and avoids influence of injection. Also, the use of siRNA is more specific comparing to use of antagonist. Another design which also gives potential is by the use of myoblast and fibroblast cell-lines or primary culture from muscle and connective tissue of ST36. It was demonstrated by Goldman *et al.*[19] that ATP is released during acupuncture at ST36. Therefore, it is logical that the release of ATP is detectable when mechanical force is applied to muscle cells or fibroblast cultured on soft matrix. If stimulation of TRPV1 is related to ATP release as reported,[21] then the ATP release should also be detectable when capsaicin is added to medium. Conversely, the release of ATP by mechanostimulation is reduced when antagonist is added to medium. This in vitro model, if stable, is not only suitable for further tests on CWP with calcium image but could also serve as an in vitro model for acupuncture research. Also, injecting mixture of capsaicin with TRPV1 antagonist may give alternative proof about capsaicin's analgesic effect.

To functionally link TRPV1 and CWP components with analgesic effect, co-injection of capsaicin and CWP component antagonist is suggestive. If analgesic effect from capsaicin is block at least partially by CWP component antagonist, this can further prove CWP is downstream of TRPV1 activation.

Chapter 6 : Conclusion and Perspective

As a conclusion, TRPV1 is expressed in neural and non-neural cells at acupoints, and its activation may replicate the effect of acupuncture. Also, both the neural and the non-neural cell initiated sensing pathways may participate in conveying signal from mechanostimulation to nervous system. These results may lead to clinical studies of capsaicin application to acupoints. Perhaps the application of capsaicin or other TRPV1 agonists will represent an additional treatment option and enhance the effect of acupuncture. Given the capabilities of TRPV1 for receiving mechanical and thermal stimulations, as in acupuncture and moxibustion (thermal stimulation in traditional Chinese medicine), more extensive studies on the role of TRPV1 during acupuncture should be performed to determine whether it is an “acupuncture-responding channel.”

Chapter 7 : Reference

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Chapter 8 : Acknowledgement

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Thank you all! Thank You!