Research article

**In vivo voltage-sensitive dye imaging of the insular cortex in nerve-injured rats**

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**HIGHLIGHTS**

- Excitability of the IC to peripheral stimulation is changed after nerve injury.
- Optical signals in the IC increased as the stimulation intensity was elevated.
- Plastic changes in the IC could be related to nerve injury-chronic pain.

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**ABSTRACT**

The insular cortex (IC) is a pain-related brain region that receives various types of sensory input and processes the emotional aspects of pain. The present study was conducted to investigate spatiotemporal patterns related to neuroplastic changes in the IC after nerve injury using voltage-sensitive dye imaging. The tibial and sural nerves of rats were injured under pentobarbital anesthesia. To observe optical signals in the IC, rats were re-anesthetized with urethane 7 days after injury, and a craniectomy was performed to allow for optical imaging. Optical signals of the IC were elicited by peripheral electrical stimulation. Neuropathic rats showed a significantly higher optical intensity following 5.0 mA electrical stimulation compared to sham-injured rats. A larger area of activation was observed by 1.25 and 2.5 mA electrical stimulation compared to sham-injured rats. The activated areas tended to be larger, and the peak amplitudes of optical signals increased with increasing stimulation intensity in both groups. These results suggest that the elevated responsiveness of the IC to peripheral stimulation is related to neuropathic pain, and that neuroplastic changes are likely to be involved in the IC after nerve injury.

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1. Introduction

Chronic pain refers to prolonged pain that remains after recovery from peripheral nerve damage [1]. Recent studies have speculated that plastic changes induced by nerve injury in pain pathways may be the main cause of chronic pain [1–4]. A better understanding of the six brain areas related to pain processing that make up the pain matrix, namely, the anterior cingulate cortex, insular cortex (IC), primary somatosensory cortex (S1), secondary somatosensory cortex (S2), prefrontal cortex (PFC), and thalamus is important for treating chronic pain, since pain is both a subjective sensation and an emotional experience that requires integrated multidimensional processing in pain-related brain networks [5–7]. In fact, several studies have reported the existence of structural or functional plasticity in the ACC [8,9], S1 [10], and PFC [11] in neuropathic and chronic pain models.

Although the IC has been conventionally regarded as a gustatory cortex, it was recently reported that the IC may play a critical role in processing and modulating pain sensation [12–15]. Evidences from clinical and animal studies suggest that the IC is related to the affective-motivational dimension of pain, because patients with IC lesions exhibit pain asymbolia, an absence of withdrawal response...
to painful stimuli although the primary sensation is not affected [16]. In addition, rats with IC lesions show significantly decreased neuropathic and inflammatory pain behavior [17,18]. Numerous recent reports have suggested that the plasticity of pain-related brain areas contributes to chronic pain states [1]. Consistently, we previously observed plasticity-related signaling in the IC induced by nerve injury [19]. Nevertheless, there are relatively few studies about nerve injury-related plastic changes in the IC and their contribution to chronic pain.

Optical imaging using voltage-sensitive dyes is a functional brain imaging technique that enables visualization of neuronal activity of large populations and changes in membrane potential [20]. Owing to the high spatiotemporal resolution of this technique, several studies have reported excitation propagation patterns in several brain areas [21–25]. For example, we previously demonstrated that S1 cortical excitability is modified following nerve injury [26]. However, there have been no reports on functional plastic changes in the rat IC after nerve injury using in vivo optical imaging. Thus, the purpose of this study was to investigate excitation patterns and changes related to neuroplasticity in the IC after nerve injury using optical imaging.

2. Materials and methods

2.1. Animals

All experimental procedures in this study were conducted in accordance with the National Institute of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the Yonsei University Health System. Male Sprague-Dawley rats (Koatec, Pyeongtaek, Korea, 200–280 g) were allowed to acclimate for a period of 7 days after arrival and were accommodated in the temperature-controlled room with food pellets and water provided ad libitum.

2.2. Neuropathic surgery

Under sodium pentobarbital (50 mg/kg, i.p.) anesthesia, branches of the left sciatic nerve were exposed by dissecting the skin and biceps femoris muscle. The tibial and sural nerves were tightly ligated and transected, while the common peroneal nerve was left intact [27]. Muscle and skin layers were sutured and a drop of lidocaine was applied to the wound. The sham control group underwent the same operation without any nerve damage.

2.3. Behavioral test for mechanical allodynia

A mechanical threshold test was used to confirm the development of neuropathic pain. The method of the mechanical allodynia test was described in our previous study in detail [19]. Briefly, a blind test was conducted to observe the mechanical threshold on post-operative days (PODs) 1, 3, and 7. Rats were placed on an elevated metal mesh floor under rectangular-shaped transparent domes and familiarized to the test conditions for 15 min. Mechanical withdrawal thresholds were assessed by applying an electronic von Frey filament (UGO Basile, Varese, Italy) to the medial dorsal paw area of nerve-injured left hind paws. The mechanical withdrawal threshold was tested eight times, and the results excluding maximal and minimal values were averaged. The mechanical force was recorded when a positive withdrawal response of the hind paw occurred, defined as licking or flicking of the left paw.

2.4. In vivo optical imaging

Rats (sham and nerve injury group, n = 5 and n = 7 respectively) received dexamethasone sulfate (1 mg/kg, ip) one day before the optical imaging experiment to reduce swelling of the cortex. Seven days after nerve injury, rats were anesthetized with urethane (1.25 g/kg, i.p.) and given atropine (5 mg/kg, i.p.) to prevent mucus secretion. Rats were placed on their side in a custom-made stereotaxic frame such that they were lying in a lateral position, which afforded access to the IC located in the anterolateral aspect of the rat brain. An ophthalmic lubricant ointment was applied to both eyes to prevent desiccation. Heart rate was monitored by electrocardiography and body temperature was maintained at 36 °C with a rectal probe and heating pad system (Homethermic Blanket Control Unit, Harvard Apparatus, Holliston, MA, USA). Prior to craniectomy, lidocaine was applied to the right temporal muscle and the skin contralateral to the nerve injury. Craniectomy was performed to visualize the IC after preparation steps. The skin overlaying the temporalis muscle, the muscle, and zygomatic arch were carefully resected to prevent excessive bleeding. Finally, the cortex was stained using a voltage-sensitive dye (VSD; di-2-ANEPQ, 50 μg/mL in saline, Molecular Probes, Eugene, OR, USA) after the dura was removed to expose the surface of the cortex. The cortex was carefully rinsed with saline one hour after staining.

The optical imaging method was performed as described previously [26,28]. Briefly, the imaging site was focused on the IC surface with the camera axis perpendicular to the IC. The fluorescence of the dye was detected using an optical imaging system consisting of a high resolution CCD camera (Brainvision Inc., Tokyo, Japan) equipped with a dichroic mirror with a 510–550 nm excitation filter and 590 nm absorption filter. A tungsten halogen lamp (150 W) was used for excitation of fluorescence. The imaging area was 6.4 × 4.8 mm² and consisted of 184 × 124 pixels.

2.5. Electrical stimulation

A pair of stainless steel electrodes was implanted in the peripheral receptive field of the left hind paw where the electronic von Frey filament was applied during behavioral testing. The receptive field was stimulated with a square pulse (width: 0.1 ms, interstimulus interval: 5 s, intensity: 0.06, 1.25, 2.5, and 5.0 mA) using a stimulus isolation unit (World Precision Instruments, Sarasota, FL, USA). During each trial, the change in fluorescence intensity was measured for approximately 940 ms. The recording surface was placed under an optical microscope (Leica Microsystems Ltd., Heerbrugg, Switzerland) equipped with a 1 × objective and 1 × projection lens. Optical signals were acquired at a rate of 3.7 ms/frame and averaged 30 times using an optical imaging recording system (MiCAM02, Brainvision Inc.). Optical imaging acquisition was triggered by electrocardiogram signals using a stimulus/non-stimulus subtraction method. After optical imaging, rats were euthanized by over dose of urethane.

2.6. Data analysis

In order to normalize the value of each pixel, the ratio of the intensity of fluorescence (ΔF) in each pixel relative to the initial fluorescence intensity (F) was expressed as the fractional change (ΔF/F). Amplitudes and excitatory areas of optical signals were measured using a spatial filter (9 × 9 pixels) to reduce artifacts caused by vibration and brain movements. Data were collected and illustrated using BV Analyzer software (Brainvision Inc.).

Using captured images, fractional changes in optical signals (optical intensity) and areas of activation were quantified. Changes in optical intensity in the IC were determined as the ratio of change in the intensity of fluorescence (ΔF) to the initial intensity of fluorescence (F) expressed as percent fractional change (ΔF/F × 100). In order to analyze the area of activation, a region of interest (ROI) was delineated using a black dashed-line (circle of 20 pixel radius) as shown in Fig. 2A. The activated area was defined as the activated
pixel number of the ROI/total pixel number of the ROI × 100. The optical intensity and the activated area were analyzed using BV Analyzer software (Brainvision Inc.).

Data are presented as the mean ± standard error of the mean (SEM). Differences in intensities of optical signals and areas of activation were analyzed with unpaired t-tests. P values less than 0.05 were considered significant.

3. Results

3.1. Development of neuropathic pain

Mechanical allodynia was elicited by injuries of the two major branches (the sural and tibial nerves) of the sciatic nerve on PODs 1, 3, and 7 (Fig. 1). The mechanical threshold of the nerve-injured group decreased on PODs 1, 3, and 7 relative to the sham group (P < 0.01, unpaired t-test).

3.2. Representative spatiotemporal patterns of optical signals

To distinguish among optical signals from the IC, the lateral view of the exposed cortex was delineated according to previous reports (Fig. 2A) [29,30]. Representative optical images were obtained by electrical stimulation (1.25 mA) of the contralateral hind paw in sham and nerve-injured rats (Fig. 2B and C). Images of the left column were color-coded to show optical signals induced by stimulation according to the fractional changes in reflected light intensity (%ΔF/F). Wave forms were used to show the optical responses at specific points in the ROI (red dots) in the IC. Whereas the IC of the sham-operated rats rarely showed a response, the IC of neuropathic rats was extensively activated in response to peripheral electrical stimulation (Fig. 2C).

3.3. Optical signals in response to different intensities of electrical stimulation

A time course of optical signals induced by different intensities of electrical stimulation was used to show the differences between neuropathic and sham-operated rats (Fig. 3). The color-coded activated area and wave forms of sham-operated and nerve-injured rats are shown in Fig. 3A and B, respectively. Although activated signals were not detected in the IC of the sham group after 0.6 and 1.25 mA electrical stimulation, 1.25 mA stimulation led to activation in the nerve-injured group. Electrical stimulation intensity of 2.5 and 5.0 mA led to excitation in both sham-operated and nerve-injured rats. In addition, the nerve-injured rats exhibited a more extensive and longer duration of excitation than sham-operated rats. The S2 was obviously activated by electrical stimulation due to the way divisions of the body are represented in the sensory cortex, the so called ratunculus. However, activation in the S2 area was excluded from our analysis because it was not included in the region of interest.

3.4. Quantitative analysis of changes in optical signals after nerve injury

Peak amplitudes and the activated area are represented in Fig. 4A and B, respectively. The peak amplitude evoked by 5.0 mA electrical stimulation of nerve-injured rats was increased compared to sham-operated rats (Fig. 4A, P < 0.05). The activated area evoked by 1.25 and 2.5 mA electrical stimulation in nerve-injured rats was also increased compared to sham-operated rats (Fig. 4B, P < 0.05). On the other hand, there was no significant difference between nerve-injured and sham groups following 5.0 mA stimulation. These results suggest that peak amplitude is more sensitive to high stimulation intensity and that the activation area is likely to become saturated at relatively low stimulation intensities. Sham-operated and neuropathic rats tended to exhibit an increased amplitude and activated area following electrical stimulation at different intensities ranging from 0.6 to 5.0 mA. In addition, neuropathic rats displayed a more sensitive response to increasing stimulation intensities (Fig. 4A and B).

4. Discussion

The purpose of this study was to determine whether the IC is a site of pain information processing, and if it exhibits plasticity after nerve injury. This is the first study to demonstrate optical signals in the IC induced by peripheral electrical stimulation in a neuropathic pain model. Specifically, we observed optical signals of the IC using VSD in response to electrical stimulation of the contralateral hind paw. Amplitudes and activated areas of optical signals in the IC were increased as the intensity of electrical stimulation was elevated. In addition, we observed modification of excitability in the IC after nerve injury. Together, these results shed light on the pain processing function of the IC in chronic pain states related to nerve injury-induced plastic changes.

4.1. Optical signals of the IC induced by electrical paw stimulation

A large number of neuroimaging studies have reported that noxious stimuli evoke activation of specific brain areas comprising the so called pain matrix [6]. Activation of the S1 and S2 is consistently observed in response to nociceptive stimulation in fMRI, PET, EEG, and MEG studies [7,31,32]. These areas have specialized sub-functions and handle the sensory-discriminative aspects of pain processing. On the other hand, emotional aspects of pain are represented in the limbic areas such as the ACC and IC [1,12]. In fact, the human S2 and posterior IC respond differently to CO2 laser stimulation as determined by electrode recording [33]. Specifically, whereas the S2 is sensitive to changes in the intensity of sub-threshold pain stimuli, the IC is sensitive to variations in the level of painful stimuli. According to an animal study, the S2 and IC are activated by hind paw electrical stimulation [17]. Consistent with this observation, our data showed that S2 and IC were activated by peripheral electrical stimulation. Interestingly, while the S2 was activated by stimuli ranging in intensity from low to high, the IC was only activated by high intensity stimulation. A high-resolution fMRI study showed that the IC responds to painful infrared laser mechanical stimulation [34]. In addition, Craig et al. [35] used PET to observe cooling stimuli-evoked contralateral activation of the IC. Taken together, these reports suggest that the IC may be specifically activated by painful stimuli. In addition, our data provided the first evidence that the IC can be activated by electrical stimulation in a neuropathic model using in vivo optical imaging. Given the number
of pain-related studies based on fMRI studies and recent critiques doubtful of their validity due to an error in fMRI software [36], the results of the present study are useful for confirming past reports that the IC is indeed part of the pain matrix area.

4.2. Excitability of the IC in chronic pain

The IC is located deep inside the lateral fissure in humans and, in addition to gustatory functions, plays a critical role in the interpretation of the emotional aspects of pain [37]. Several human studies have provided insight into the pain-related functions of the IC. Specifically, lesions in this region cause pain asymbolia, a state in which a patient is able to recognize painful stimuli but does not avoid because the patient cannot interpret this stimuli as painful stimuli [16]. Human electrode recording and fMRI studies indicate that both acute and chronic pain result in activation of the IC, and also that direct stimulation of the IC can cause pain sensation [12,33,38–40]. Patient’s with irritable bowel syndrome and accompanying hyperalgesia exhibit increased activity of the IC and ACC [41]. Likewise, an EEG study reported that patients suffering from chronic neurogenic pain have enhanced spontaneous activity, such as high and low theta frequencies, from pain matrix areas including the IC compared to healthy individuals [42].

In rodents, lesions to the IC reverse neuropathic pain states to normal states, but do not affect non-nociceptive mechanical thresholds [17,43]. Jasmin et al. [13,15] showed that the IC has a key role in pain information processing and pain modulation. Specifically, the rostral agranular IC is efferrant and afferent connections to nociceptive processing areas and can change the pain threshold using GABA neurotransmission. However, there have been relatively few studies regarding excitability in the IC in chronic pain models. In the present study, we found that optical signals of the IC were increased in the neuropathic and sham group as peripheral stimulation increased. On the other hand, the neuropathic group showed high optical signals in response to low stimulation while the sham group showed low optical signals in response to high intensity stimulation, which was consistent with the human imaging studies described above [41,42].

According to our study, optical intensities and activated areas may be related to neuronal activation in response to pain sensation. To date, there were no optical imaging studies that observed changes in the IC. However, we can assume the significance of the increases in optical intensity and activated area through electrophysiological and imaging studies. For example, in a human study [33], higher scores on the visual-analogue scale (VAS) were obtained and laser-evoked potentials increased as intensities in peripheral stimulation elevated. In this case, laser-evoked potentials may correspond to optical intensity. Another study [40] reported that as the pain sensation becomes more intense, the more laser-evoked potentials in the operculoinsular and primary somatosensory areas are observed. An fMRI study using the heat/capsaicin sensitization model showed that more activated areas were observed in humans with hyperalgesia compared to controls [44]. An animal study with fMRI reported that BOLD signals and activated areas increased in brain areas including the IC in hyperalgesia rats [45].

In the present study, different intensities of peripheral stimulation showed differences in optical signals including both peak intensities and activated areas between neuropathic and sham-operated rats. The peak amplitude evoked by 5.0 mA electrical stimulation of nerve-injured rats was increased compared to sham-operated rats. The activated area evoked by 1.25 and 2.5 mA electrical stimulation in nerve-injured rats was increased compared to sham-operated rats. However, there was no significant difference in activated areas between nerve-injured and sham groups following 5.0 mA stimulation. Therefore, there may be some discrepancies between peak intensities and activated areas in our data. It can be difficult to explain these discrepancies in detail solely by this study. But we speculate that the activated area in neuropathic rats is likely to become readily saturated at relatively low stimulation intensities. In addition, the activated area in sham controls approaches the saturation level in an intense stimulation (5.0 mA) because the region of interest (ROI) covers a relatively smaller area in the IC. In contrast, it seems that the peak amplitude is not saturated readily and shows a more sensitive response to increasing stimulation intensities in neuropathic rats.

4.3. Plasticity of pain information processing brain areas in chronic pain states

There are a variety of plastic changes in pain-related brain areas in the chronic pain state. Patients with chronic back pain due to neuropathic and non-neuropathic causes have decreased gray matter density in the central nervous system [46,47]. According to several animal studies, functional and structural plastic changes in the S1, S2, and PFC have been reported [48]. The ACC is considered to be
Fig. 3. Sequential images and time course of optical signals in sham (A) and neuropathic (B) rats. Optical activity was induced using different intensities of electrical stimulation to the hind paw in sham and neuropathic rats. Electrical stimulation was applied to the hind paw at 0 ms (red arrow). Lower panels of both (A) and (B) represent the waveforms of optical signals activated by different intensities of electrical stimulation. S2 was obviously activated in the hind paw area of the ratunculus by peripheral electrical stimulation, but was excluded from our analysis because it was not in the ROI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
related to the emotional aspects of pain as part of the limbic system [1]. Recently, Li et al. [8] demonstrated that nerve injury-induced plasticity in the ACC contributes to chronic pain, and that elimination of this plasticity can reverse the chronic pain state [5]. In addition to the ACC, several studies have observed that peripheral nerve injury induces functional plasticity in the IC related to NMDA and AMPA receptors [49,50]. According to our previous study [19], changes in plasticity-related molecules were observed after nerve injury and AMPA receptor subunits. This selective inhibitor of PKMζ microinjection into the IC decreased mechanical allodynia. Taken together, these results suggest that plasticity of the IC induced by nerve injury may contribute to the chronification of neuropathic pain. In line with these reports, we observed in the present study that the amplitudes and activated areas of optical signals to the peripheral stimulation were significantly increased in the neuropathic pain group compared to the control group. We speculated that this phenomenon was due to nerve injury-induced plasticity in the IC. However, as there are few reports about nerve injury-induced plasticity of the IC and its contribution to chronic pain, it will be necessary to explore the IC in greater detail in future studies.

5. Conclusions

Using an optical imaging technique we demonstrated two major changes in activation amplitude and area in the IC elicited by peripheral electrical stimulation after nerve injury. Because optical imaging using VSD has a high spatial and temporal resolution, this technique is suitable to detect direct responses of the IC. Our data also demonstrated the presence of nerve injury-induced plasticity in the IC, and suggested that the IC can be a target of chronic pain modulation. Further studies on the detailed characteristics of plasticity in the IC will be necessary to elucidate the specific mechanisms of nerve injury-induced plastic changes in the IC.

Conflicts of interests

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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