Neuroplastic changes in insular cortex induce neuropathic pain like behavior

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Directed by Professor Jin Woo Chang

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우선 바쁘신 시간을 내주셔서 논문 심사를 해주신 김정훈 심사위원장짐과, 이배환 심사위원님께 깊은 감사의 말씀드립니다. 2년 동안 학위 논문을 완성하기 까지 많이 부족한 저를 지도해 주시고 격려해주신 장진우 교수님, 실험에 있어서 많은 조언을 주신 정현호 교수님, 장원석 교수님께 감사의 인사를 드리고 싶습니다. 또한 학위 논문을 위한 실험을 진행함에 있어 실험 설계 및 결과를 도출 하는 전 과정에 있어서 가장 큰 도움을 주신 고진수 선생님께도 감사의 말씀 드립니다. 실험실 생활을 하면서 부족한 저에게 같이 실험을 하면서 많을 것을 배울 수 있었던 이지현 선생님, 신재우 선생님께 감사드립니다. 항상 힘든 일이 있을 때마다 아낌없이 조언해준 공찬호 선생님 덕분에 마무리를 잘 할 수 있었던 것 같습니다. 감사드립니다. 마지막으로 석사 과정을 끝까지 마칠 수 있도록 격려해주신 가족들에게 감사의 말씀을 드리며 이 글을 마칩니다.

2018 년 8 월

윤민식 드림

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ABSTRACT

Neuroplastic changes in insular cortex induce neuropathic pain like behavior

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Neuropathic pain involves abnormal sensitivity of the central nervous system. Although the mechanisms underlying its development of sensitization remains unclear, recent studies have reported that neuroplastic changes in pain circuit may be involved in hypersensitivity associated with neuropathic pain. However, it is difficult to investigate such phenomena in existing animal pain models. Therefore, I developed a novel animal model—the circuit plasticity reconstruction (CPR) model—to mimic central sensitization associated with neuroplastic changes. NMDA and Ro 25-6981 were injected into the right insular cortex of Sprague-Dawley rats, while non-noxious electrical stimulation was delivered to the left hind paw. In this study, I conducted all the procedures in 3 different experimental groups: CPR, modified CPR 1 (mod-CPR 1) and modified CPR 2 (mod-CPR 2). Mod-CPR 1 surgery involved electrical stimulation to the left hind paw with PBS injection into the insular cortex. Mod-CPR 2 surgery

involved the injection of a mixture of NMDA and Ro 25-6981 into the insular cortex, but no electrostimulation. To verify induction of sensitization and mechanical allodynia, the von Frey test was conduct with the up-down scoring method. The mechanical withdrawal threshold of the left hind paw decreased beginning 1 day after CPR modeling and persisted until day 21 comparing to the modified CPR 1 (mod-CPR 1) group (CPR: 91.68 ± 1.8%, mod-CPR 1: 42.71 ± 3.4%, $p < 0.001$). In contrast, mod-CPR 2 surgery did not induce mechanical allodynia. Neuroplastic changes in insular cortex after CPR surgery were confirmed by PSA-NCAM immunohistochemistry staining with the intensity of signal being significantly increased in the CPR group. My results demonstrated that the CPR model induced neuroplasticity within the insular cortex, leading to alterations in neural circuitry and central sensitization.

Key Words: Neuropathic pain, Insular cortex, Neuroplastic changes, Hypersensitivity, Mechanical allodynia

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I. INTRODUCTION

Drug-refractory chronic pain represents a critical challenge of medicine.[1] Neuropathic pain, which occurs due to nerve injury following physical trauma, represents a major component of chronic pain syndromes. Indeed, studies have indicated that neuropathic pain is associated with abnormal sensitivity in the central nervous system,[2,3] although the mechanism underlying the development of such hypersensitivity remains to be fully elucidated.[2,4] However, recent studies have reported that alterations in neural circuitry may be involved in hypersensitivity associated with chronic pain,[5-8] and that long-term potentiation (LTP) may play a critical role in neuroplastic changes to the connections between the peripheral and central nervous systems.[9,10] Several previous studies have demonstrated that chronic pain is associated with alterations in neuronal plasticity and neurogenesis, particularly with regard to the

density and maturation of synaptic spines in brain regions associated with pain processing.[11]

Several animal models of pain (e.g., spared nerve injury (SNI), chronic constriction injury (CCI), tibial and sural nerve transection (TST)) have been established to mimic human forms of neuropathic pain.[12-14] However, these models mainly represent the pain triggered by inflammation or neuromas in the peripheral nervous system. To address this issue, I developed a novel animal model of neuropathic pain based on plasticity changes, which I have named the "circuit plasticity reconstruction (CPR)" model.

In the present study, I focused on neuroplasticity in the insular cortex, which is well known for its role in the pain matrix[10] and high capacity for neuroplastic changes in the brain. Thus, I injected the NMDA receptor modulators, (*R*)-2-(methylamino) succinic acid (NMDA) and 1-piperidinepropanol, α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-, (αR, βS)-, (2Z)-2-butenedioate (1:1), [R-(R*, S*)]-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol maleate salt (Ro 25-6981) into the insular cortex of the rat brain in order to increase excitatory responses in post-synaptic areas. I then delivered non-noxious artificial sensory input by electrically stimulating the pre-synaptic peripheral nerves. Previous *in vitro* analyses have confirmed that this process effectively induces LTP in the central nervous system,[15] and that LTP enhances the number of NMDA receptors and NMDA synaptogenesis, thereby increasing the amount of pre-synaptic input in these regions.[16,17] I hypothesized that this procedure would enhance LTP in the insular cortex and produce changes in the pain matrix, resulting in allodynia to non-noxious tactile stimuli.

II. MATERIALS AND METHODS

1. Animals

All experimental procedures of the present study were conducted in accordance with the guidelines of the Ethical Committee of the International Association for the Study of Pain, and were approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University Health System. A total of 25 male Sprague-Dawley rats (180-200 g) were used in this study. Rats were housed three per cage under a 12-hr light/dark cycle (lights on between 07:00 and 19:00) and allowed access to food and water *ad libitum*.

2. Surgical procedures for CPR modeling

Rats were anesthetized via an intraperitoneal injection of pentobarbital (40 mg/kg, Hanlim Pharm, South Korea) and positioned in a stereotaxic frame. Atropine (0.1 mL; Huons, South Korea) was administered 10 min prior to pentobarbital administration to ensure the stability of anesthesia. A mixture of 2 mM NMDA (Sigma, M3262, 1 μL) and 0.6 mM Ro-25-6981 (Sigma-Aldrich, SML0495, 0.3 μL) was injected into the right rostral agranular insular cortex (RAIC, AP: 1.0 mm, LM: +5.0 mm, DV: -5.5 mm).[18] Rats of the mod-CPR 1 groups were injected with 1.3 μL of phosphate buffered saline (PBS) in the same target region. A pair of electrodes was then inserted at the lateral edge of the plantar area of the left side hind paw, and electrical stimulation (350 μA,

100 Hz, biphasic pulse: 8 ms) was delivered for 3 hr under anesthesia (Fig.1). Lidocaine (0.1 mL) was injected into the right hind paw to diminish unnecessary sensory input.

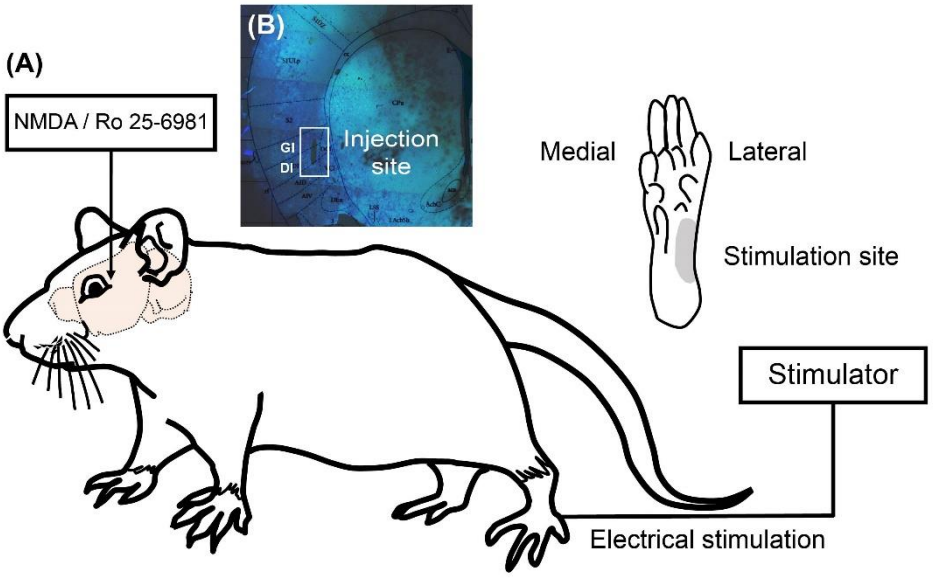

Figure 1. Experimental set up. (A) Schematic diagram of circuit plasticity reconstruction (CPR) modeling (B) Tile scan image of the injection site (magnification: 5x, GI: granular insular cortex, DI: dysgranular insular cortex).

3. von Frey filament test

Following recovery from surgery, rats underwent von Frey filament testing[19] on post-operative days 1-21 (every 2 days). Rats were positioned inside of a hexahedron-shaped cage (8 x 10 x 12 cm) on a testing frame composed of mesh wire. To ensure the precision of measured responses, rats were adapted to the testing frame for at least 30 min. Testing was performed at the lateral edge of both hind paws using the up-down method.[20]

4. Immunohistochemistry

After 3 wk of CPR surgery, rats were intracardially perfused with saline and fixed with 4% paraformaldehyde (PFA). Brains were then carefully extracted, frozen (-20 ℃), and prepared for sectioning. The brains were sliced into 30 μm coronal sections using a cryostat and preserved in cryoprotectant solution (0.1 M PBS, 30% sucrose, 1% polyvinylpyrrolidone, 30% ethylene glycol). Sections were blocked with 10% normal goat serum in PBS with 0.3% Tween 20 (PBS-T, pH 7.4, CNP001-1000, Cellnest) at 25 ℃ for 2 hr. Tissues were incubated overnight with the primary antibody, mouse anti-PSA-NCAM (polysialylated neural cell adhesion molecule), clone 2-2B (1:100; EMD Millipore, MAB5324, CA, USA) at 4°C. Tissues were then incubated with the secondary antibody, goat anti-mouse immunoglobulin M (IgM) (μ-chain-specific) antibody conjugated with FITC (1:200; EMD Millipore, AP128F, CA, USA) at room temperature for 90 min. For early growth response protein 1 (Egr-1) immunostaining,

mouse anti-Egr-1 (B-6) (1:00, Santa Cruz biotechnology, SC-515830,) was used as the primary antibody and goat anti-mouse immunoglobulin G (IgG) with Cy 5 florescence (1:200, Life Technology, A10524, OR, USA) was used as secondary antibody following the same experiments protocols. All sections were mounted with DAPI mounting solution (Vector, H-1200), and fluorescent images were obtained using a confocal microscope (LSM-700, Carl Zeiss). Additionally, spinal cords were coronally sectioned using a cryostat (thickness: 10 μm) and stored at -20°C in a cryoprotectatnt solution. Spinal cord sections (level: L4-L6) were mounted on slide glasses. Spinal cord tissues were incubated overnight in rabbit anti-GAD 65/67 (1:500, Millipore, AB1511, CA, USA) at 4 ℃. Tissues were subsequently incubated in goat anti-rabbit AF 488 (1:500, Invitrogen, A11008, OR, USA) for 90 min at room temperature.

Images were analyzed by using ZEN (Blue edition, Carl Zeiss). PSA-NCAM and Egr-1 positive cells were quantified as the arithmetic mean intensity of immunoreactive area per field of view and values are presented as mean and SEM (n=4 per each group).

5. Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). GraphPad Prism 5 (GraphPad Software, Inc.) was used to create graphs and perform all statistical analyses. Two-way analyses of variance (ANOVA) followed by Tukey post hoc tests were used to compare mechanical withdrawal threshold between the groups. Repeated-measures ANOVA followed by Tukey post hoc tests were used to

analyze the sensitivity effect of each group. One-way ANOVA followed by Tukey post hoc test was used to analyze the intensity of PSA-NCAM and Egr-1 immunostaining and percent change in threshold reduction.

III. RESULTS

1. CPR modeling causes central sensitization

CPR modeling with a mixture of NMDA/Ro 25-6981 and electrical stimulation, induced intense hypersensitivity in rats. Mechanical withdrawal threshold of the left hind paw significantly decreased compared to pre-operative values, beginning on post-operative day 1, and persisting for 3 wk (Fig. 2A, CPR group; $p < 0.001$ (1 d to 21 d), n = 11). The mechanical threshold in the mod-CPR 1 group decreased relative to pre-operative values, but the degree of change was less than in the CPR group (Fig. 2B, mod-CPR 1 group; $p < 0.05$ (7 d, 19 d and 21 d), n = 7). However, in the mod-CPR 2 group, a significant decrease in threshold values was only detected on the first day after surgery (Fig. 2C, mod-CPR 2 group; $p < 0.05$ (1 d), n=7). These data indicated that CPR surgery only induces mechanical allodynia, indicated by a persistent decrease in withdrawal threshold from post-operative day 1 to day 21.

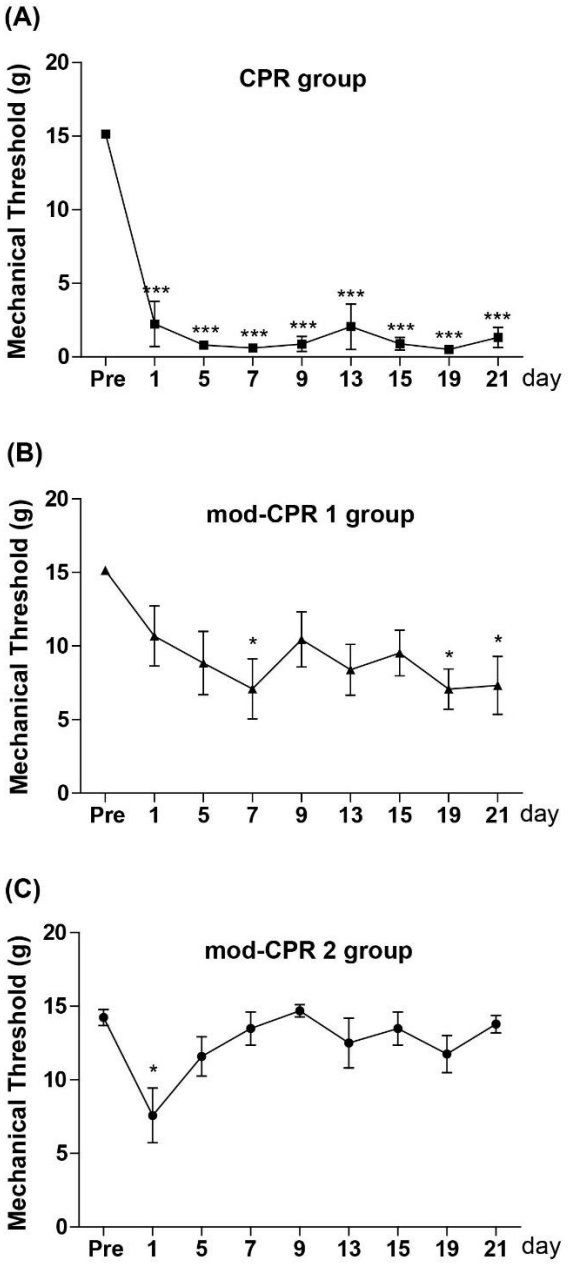

Figure 2. CPR surgery induced prolonged hypersensitization of the mechanical withdrawal threshold. (A) The mechanical withdrawal thresholds of the left hind paw

were significantly decreased in the CPR group on all post-operative days compared with pre-operative threshold values. Hypersensitivity in the left hind paw persisted for 3 wk after CPR surgery (n = 11). (B) In mod-CPR 1 group, significant differences in the mechanical withdrawal threshold in left hind paw were observed at the day 7, day 19 and day 21 (n = 7). (c) In the mod-CPR 2 group (n = 7) a significant decrease from pre-operative threshold values was only seen on post-operative day 1. Bars represent the mean ± SEM. Repeated measured ANOVA with Tukey *post hoc* tests were used to determine statistically significant within groups differences. $^{*}p < 0.05$, $^{***}p < 0.001$.

2. CPR induced hypersensitivity

To confirm the hypersensitivity effect of different surgeries, I compared withdrawal threshold values in both paws, for each group. Withdrawal threshold values were lower in CPR groups compared to mod-CPR 1 groups, from the first operative day to day 19 (Fig. 3A and 3B, CPR group vs. mod-CPR 1; $p < 0.001$ (9 d and 15 d), $p < 0.01$ (1 d, 5 d and 13 d), $p < 0.05$ (7 d and 15 d) n = 7 in each group). However, the mechanical withdrawal threshold of the right (ipsilateral to injection hemisphere) hind paw did not significantly differ between these two groups (Fig. 3B). The withdrawal threshold of the CPR group was reduced by an average of 91.68 ± 2.0% and 42.71 ± 3.4% respectively (Fig. 4; $p < 0.001$). Consequently, only the CPR group showed a significant decrease in from pre- to post-operative threshold ($p < 0.001$).

To confirm the role of electrical stimulation, I designed a modified CPR 2 (mod-CPR 2) surgery, in which no intracutaneous electrical stimulation was applied to the lateral plantar side of the left hind paw. Although the withdrawal threshold of the left side had decreased one day after surgery in the mod-CPR 2 group, this decrease was resolved by day two, indicating that the mechanical threshold reduction was not induced by long-term hypersensitivity. However, in the CPR group, hypersensitivity persisted from day one to day 21 (Fig. 3C and 3D, CPR vs mod-CPR 2; $p < 0.001$ (5 d, 7 d, 9 d, 13 d, 15 d, 19 d and 21 d) $p < 0.01$ (1 d), n = 7 in each group). The average percent reduction in the withdrawal threshold in the CPR group was 91.68 ± 1.8% in

the CPR group and 18.34 ± 5.1% in the mod-CPR 2 group (Fig. 4; $p < 0.001$). The difference between pre- and post-operative values was significantly greater in the CPR group compared to the mod-CPR 2 group. Moreover, no significant differences in hypersensitivity of the right hind paw were observed between the two groups (Fig. 3D). The values of both hind paws in the mod-CPR 1 and mod-CPR 2 group only differed significantly in the left side on day 7 and day 21 (Fig. 3E, mod-CPR 1 vs mod-CPR 2; $p < 0.05$ (7d and 21d), n = 7), whereas no differences were seen in the right hind paw (Fig. 3F). These data indicate that the reductions in withdrawal threshold induced by mod-CPR 1, were not substantive.

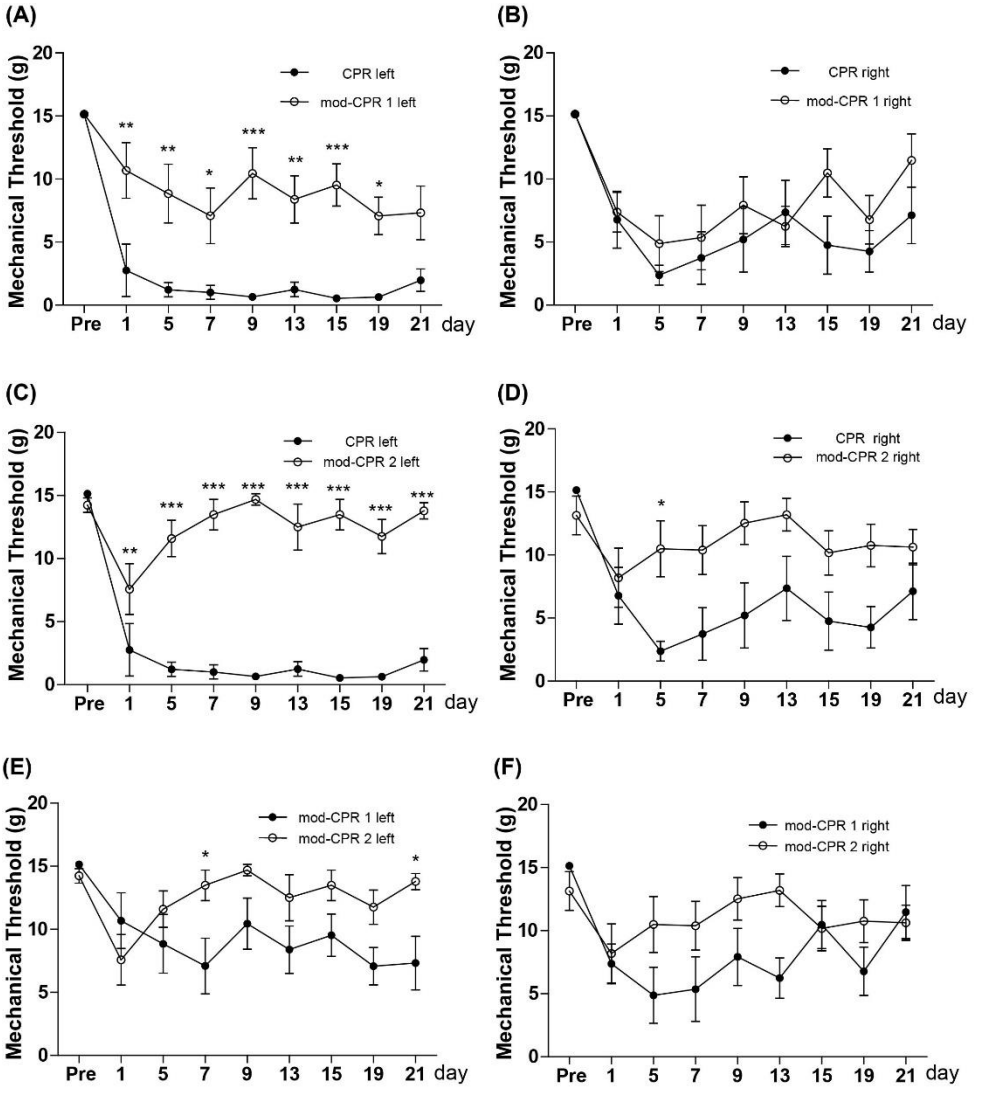

Figure 3. Withdrawal threshold values in the CPR, mod-CPR 1 and mod-CPR 2 groups. (A) and (B) Compared to the mod-CPR 1 group, mechanical withdrawal thresholds of the left hind paw were significantly decreased in CPR group on post-operative days 1

to day 19. Hypersensitivity in the left hind paw persisted for 3 wk after CPR surgery (n = 7). No significant differences in the mechanical withdrawal threshold of the right hind paw were observed between the CPR and mod-CPR 1 groups (n=7). (C) and (D) Compared to the mod-CPR 2 group, mechanical withdrawal thresholds of the left hind paw were significantly decreased in CPR group on post-operative days 1 to 21. Hypersensitivity in the left hind paw persisted for 3 wk after CPR surgery (n = 7). No significant differences in the mechanical withdrawal threshold of the right hind paw were observed between the CPR and mod-CPR 2 groups (n = 7). (E) and (F) Compare to the mod-CPR 2 group, mechanical withdrawal thresholds of the left hind paw were significantly decreased in mod-CPR 1 group on post-operative from days 7 and 21 (n = 7). No significant differences in the mechanical withdrawal threshold of the right hind paw were observed between the mod-CPR 1 and mod-CPR 2 groups (n = 7). Bars represent the mean ± SEM. Two-way ANOVA with Bonferroni *post hoc* tests were used to determine statistically significant differences between the groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

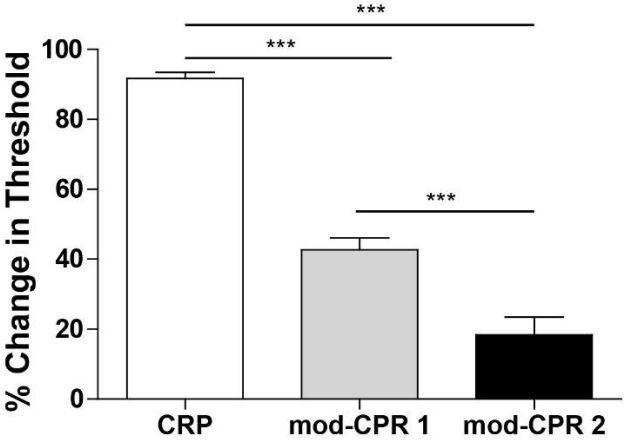

Figure 4. Average percent reduction in mechanical withdrawal threshold after each type of surgery. Compare to the mod-CPR 1 group, the percent reduction in mechanical withdrawal thresholds in the left hind paw were significantly different from that of the CPR model group (CPR group: -91.68 ± 2.0%, mod-CPR 1 group: -42.71± 3.4%). The percent reduction was significantly different in the CPR and mod-CPR 2 group (mod-CPR 2; -18.34± 5.1%). Bars represent the mean ± SEM. One-way ANOVA followed Tukey *post hoc* test was used to determine statistically significant differences between the groups. $^{***}p < 0.001$.

3. CPR induces neuroplastic changes in the insular cortex

Increases in PSA-NCAM levels play an important role in activity-dependent synaptic changes by enhancing synaptic strength and altering synaptic morphology, and are indicative of neuroplastic changes in adult rats.[21,22] Thus, I examined PSA-NCAM expression in the neurons of the insular cortex in the CPR, mod-CPR 1, and mod-CPR 2 groups. To analyze the immunoreactivity, I measured the mean intensity of positively stained cells per visual field. Immunoreactivity at the injection site was approximately four times more intense in the CPR group compared to the mod-CPR 1 group and six times more intense than the mod-CPR 2 group (Fig. 5G, $p < 0.001$). These results indicate that synaptogenesis and neuroplastic changes in the insular cortex only occurred in the CPR group, and not in the mod-CPR 1 or mod-CPR 2 groups. Since increased Egr-1 plays a key role in late-phase LTP, Egr-1 positive immunostaining near the insular cortex injection site was used as a marker of LTP induction. Positive Egr-1 immunoreactivity was observed in all groups, however, no significant differences in signal intensity were observed (Fig. 5H).

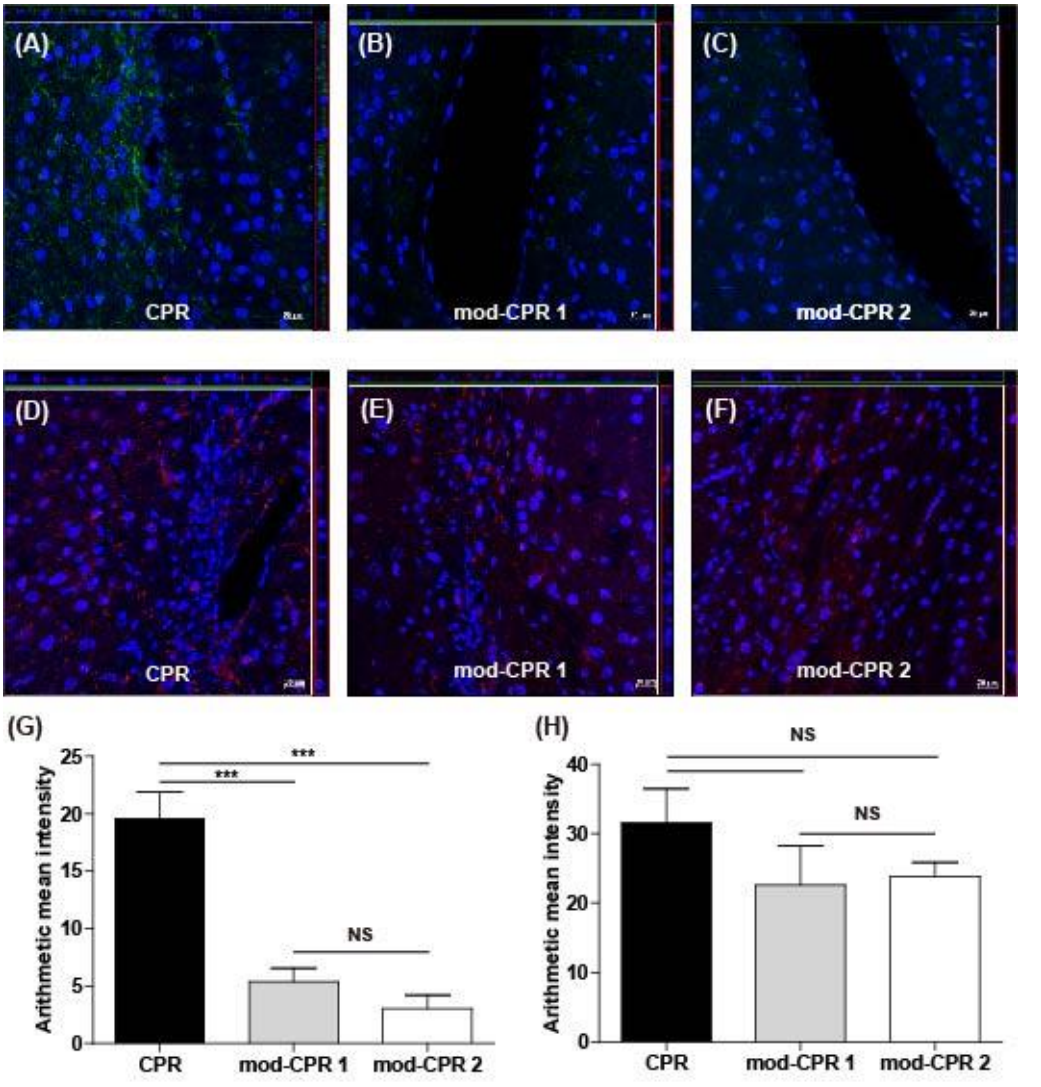

Figure 5. Immunohistochemistry images of NMDA/Ro 25-6981 injection site in insular cortex. (A), (B) and (C) CPR surgery mediated neuroplastic changes in the insular cortex. Positive staining for PSA-NCAM antibodies at the injection site in the CPR group, indicative of plastic changes in adult neurons. (G) No positive staining was

detected at the injection site in the mod-CPR 1 and mod-CPR 2 groups (z-stack image, magnification: 20X, scale bar: 20 μm). (D), (E) and (F) Egr-1, an LTP marker, was detected in all groups. Positive staining for Egr-1 antibodies at the injection site in the CPR, mod-CPR 1 and mod-CPR 2 groups. Positive Egr-1 staining is indicative of LTP inducing. (H) However, no significant difference in injection site signal intensity were detected between all groups (z-stack image, magnification: 20X, scale bar: 20 μm). The mean intensity per field of view was significantly higher in CPR group. Bars represent the mean ± SEM. One-way ANOVA followed Tukey *post hoc* was used to determine statistically significant differences between the groups. $^{***}P < 0.001$

4. CPR changes the activation of GABAergic neurons in dorsal horn

To investigate neuronal micro-environmental and morphological changes resulting from CPR surgery. I measured immunoreactivity of glutamate decarboxylase 65/67 (GAD 65/67), markers of GABAergic ineterneurons, in spinal cord tissues across all versions of the CPR surgery. Interestingly, I observed that GABAergic neurons which are in left side of dorsal horn are decreased the activation in CPR and mod-CPR 1 groups (Fig. 6A, Fig. 6B) but not in mod-CPR 2 groups (Fig. 6C).

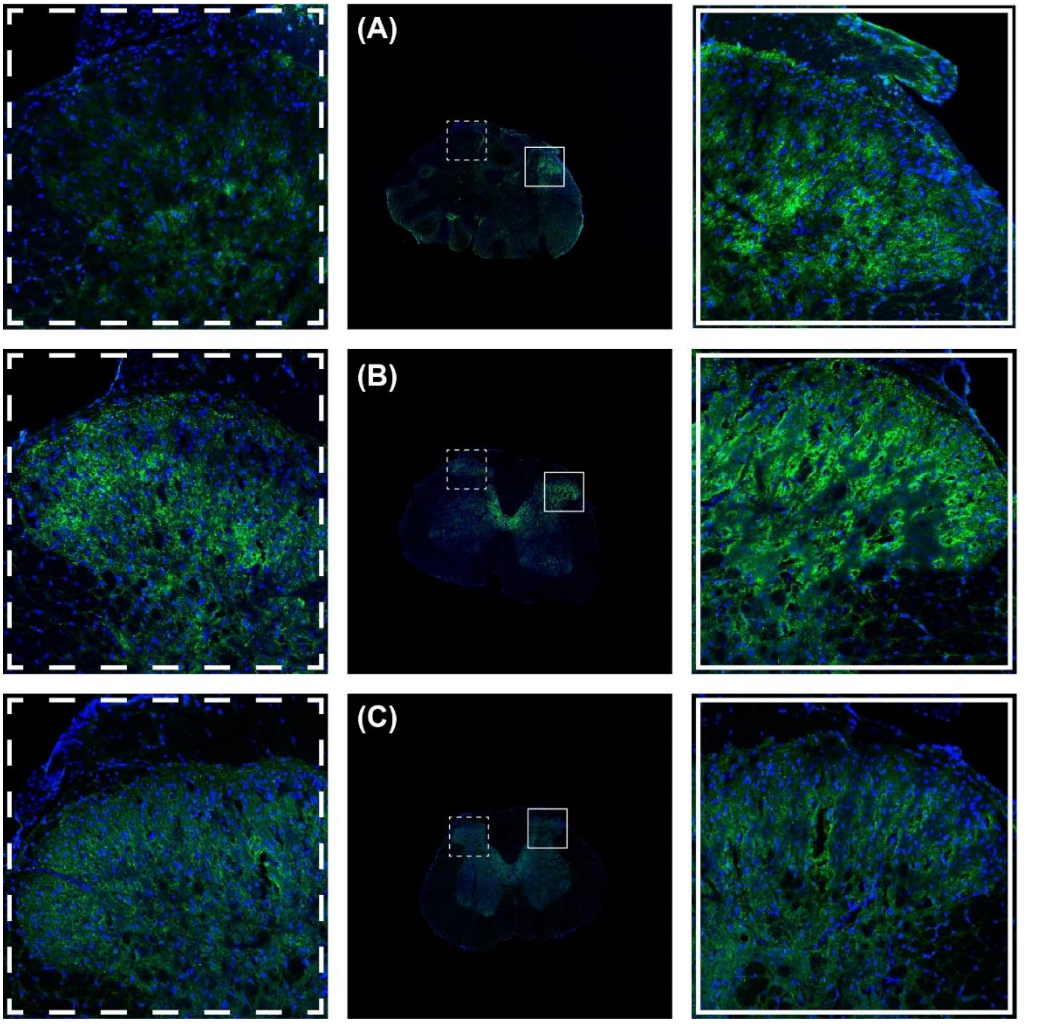

Figure 6. GAD 65/67 antibody staining of tissue from in dorsal horn of the spinal cord. In the CPR and mod-CPR 1 groups, GAD 65/67 intensity decreased in dorsal horn. However, in the mod-CPR 2 group, decreased activation of GABAergic neuron cells was not observed in the dorsal horn (magnification: 10X, tile-scan: 5x, dashed line: left side of dorsal horn, plane line: right side of dorsal horn).

IV. DISCUSSION

Neuropathic pain management is particularly challenging, and further insight into the mechanisms underlying pain production is required for the improvement of chronic pain management. Central sensitization is one of the primary characteristics of neuropathic pain.[2] Therefore, the present study investigated the association between central sensitization and neuropathic pain development. Although various animal models of pain have been developed, most are limited by a delay in pain modeling of up to two weeks following surgery. Furthermore, these models only mimic neuropathic pain based on injury to the peripheral nervous system, while effective models for examining central sensitization remain to be established. In the present study, I developed a novel animal model of central pain (CPR model), in which mechanical allodynia is accompanied by neuroplastic changes in the insular cortex, a key component of the pain matrix in the central nervous system. This model may be particularly advantageous, as it can decrease the time required for investigations of neuropathic pain and allow assessments of mechanical thresholds beginning the first day after surgery. Previous studies have reported the loss of GABAergic interneurons in the spinal cord following cord or peripheral nerve injury. GAD 65/67 are essential enzymes to produce GABA,[23] and thus, a decrease in GAD 65/67 may indirectly indicate GABAergic neuronal abnormal activation. Previous studies have also reported that the immunoreactivity of spinal cord injury (SCI)[24] and spared nerve injury (SNI)[25] were decreased in the dorsal horn, in animal pain models. Interestingly, we observed

decrease the activation of GABAergic neurons in the CPR and mod-CPR 1 groups, but not in the mod-CPR 2 group, which is consistent with the withdrawal threshold reduction tendencies of these groups. This result indicates that the decreased activation of inhibitory interneuron may be an allodynia-inducing factor in CPR surgery (Fig. 6). A clear explanation of the mechanism underlying interneuron functional abnormality after CPR surgery cannot be reached from the current results. However, I hypothesize that CPR surgery may change the micro-environment and homeostasis of glial cells in spinal cord and it can be the cause of inhibitory interneuron activity in neuronal transmission. Further studies are needed to elucidate the mechanism of neuronal and glial cells activation changes via micro-environmental modification. For this reason, I believe that our CPR model can be applied for investigations of neuropathic pain induced by hypersensitization, and in the development of novel therapeutic strategies for pain control. Thus, I aim to use the CPR model to investigate the mechanisms underlying the development of hypersensitivity in chronic pain syndromes, and to identify appropriate therapeutic approaches for drug-refractory chronic pain based on the control of neuroplastic changes.

The insular cortex was selected due to its key role in pain processing and high capacity for neuroplasticity.[26] Additionally, my group have previously reported that lesions to rostral insular cortex increases tolerance to painful stimuli and revealed a decrease in neuronal activity in the insular cortex in response to the delivery of motor cortex stimulation.[18] It has been reported that afferent responses to noxious stimulus from the somatosensory cortex first enter the caudal region of the insular cortex and are then transmitted to the rostral region. However, both the caudal and rostral insular cortex play important roles in pain processing.[27] Specifically, the rostral agranular insular cortex (RAIC) has been reported to be involved with changes to GABAergic and dopaminergic neurons. The RAIC had reciprocal connections to the cortico-cortical pathway, including infralimbic regions and the anterior cingulate cortex. Moreover, RAIC projection to the ventral forebrain influence the sensorimotor cortex and aid in the consolidation of pain related information.[28] Based on previous studies, I chose the RAIC as the target of CPR surgery to induce hypersensitivity. I hypothesized that enhanced activity in the insular cortex would lead to hypersensitivity in the pain circuitry, ultimately leading to mechanical allodynia.[29] In the present study, LTP was induced via chemical methods. Previous studies have reported that injection of NMDA and other NMDA receptor agonists effectively enhance neuronal activity.[30] However, I simultaneously injected NMDA and Ro 25-6981 to induce late phase LTP.[31] Ro 25-6981 is a selective antagonist of NMDA receptor subtype 2B (NR2B).[32,33] Although the two reagents exert opposing functions at NMDA receptors, the combination of the two results in late-phase LTP, during which the effect of potentiation is greater than that produced by NMDA alone.[31] Oh-Nishi and his colleagues suggested that the first

depression session induced by Ro 25-6981 may prime other NMDA receptors to open. When NMDA receptors open, an influx of calcium ions occurs at the synapse, mediating cell signaling and increasing LTP responses. Mallon et al. further showed that inhibition of NR2B receptors by Ro 25-6981 increases the response of NMDA receptor subtype 2A (NR2A), which may produce the influx of calcium ions necessary to induce late-phase LTP.[34] I confirmed LTP induction in the insular cortex by measuring Egr-1 levels with immunohistochemistry. Egr-1 is an immediate early gene activated by synaptic activity and plays a key role in the formation of late phase LTP. In this study, positive immunostaining was detected around the needle track.[35,36] Surprisingly, Egr-1 signal intensity did not significantly differ between groups. However, Egr-1 signal intensity was approximately 1.5 times higher in the CPR group compared to the other two groups (Fig. 5H). Based on our PSA-NCAM and Egr-1 finding, the temporal reduction in threshold observed in mod-CPR 1 and mod-CPR 2 groups can be explained by the induction of LTP in insular cortex. Moreover, electrical stimulation may induce LTP in the insular cortex in addition to injecting the combination of NMDA and Ro 25-6981. However, both LTP and simultaneous synaptogenesis are important for the induction of long lasting and intense allodynia. My immunohistochemistry data are in-line with my hypothesis that CPR surgery would induce hypersensitivity and produce neuroplastic changes in the insular cortex including synaptogenesis and LTP. Several previous studies have demonstrated that neuroplastic changes that occur in other models of neuropathic pain.[37-39] For example, neurons of the amygdala exhibit increased dendritic structure complexity seven weeks after surgery in a mouse model of tibial fracture.[39] In addition, increased dendritic

length and branching have been observed in the prefrontal cortex seven days after surgery in mice subjected to SNI.[40] Shuang et al. reported that the insular cortex of mice was activated by acute and chronic pain induced by peripheral nerve injury and that these changes were associated with an increase in NMDA receptors and alterations in surface localization.[15] Additional studies have indicated that LTP occurs following nerve injury induced with electrophysiological methods.[41,42] Taken together, these findings indicate that some regions of the brain exhibit long-term changes following injury, and that such changes may induce hypersensitization, which plays an important role in the development of neuropathic pain. My findings suggest that the proposed CPR model accelerates plastic changes in the insular cortex and enhances activation of the pain circuit. This mechanism may explain the decreased withdrawal threshold observed on the first day following operation in CPR rats, relative to the two weeks required by other animal models.

Mechanical withdrawal thresholds of the left hind paw were reduced in both the CPR and mod-CPR 1 groups. However, only the CPR group showed a significant decrease. Although the reasons for sensitization in the mod-CPR 1 group remain unclear, these effects may be due to electrical stimulation itself. Indeed, previous research groups have reported that certain forms of electrical stimulation induce pain[43-45] and that electrical stimulation of C-fibers induces central sensitization and decreases the mechanical withdrawal threshold for up to 2 days in adult rats.[11] This finding may represent the early effect of electrical stimulation during the process of central sensitization. I hypothesize that, although recovery from early-stage sensitization can be observed after 5 days, decreases in the withdrawal threshold may persist for 3 wk. I

further confirmed this with the mod-CPR 2 model, which involved CPR surgery without electrical stimulation. In mod-CPR 2 rats, the withdrawal threshold was slightly decreased on post-operative day 1 ($P < 0.05$), although complete improvement was observed by the following day. As hypersensitivity did not occur without electrical stimulation, my findings suggest that electrical stimulation induces central sensitization, while the NMDA/Ro 25-6981 mixture enhances hypersensitivity in the insular cortex to sustain this sensitization for more than 3 wk.

Morphological modifications appear when neurons undergo LTP or plastic changes. Indeed, previous studies have reported increases in the number, maturation, and complexity of dendritic spines and synapses in models of neuropathic pain model.[46] PSA-NCAM is considered a marker of adult neuroplasticity associated with neuronal migration and development.[47] Using immunohistochemistry, I was only able to detect the polysialylated form at the injection site in the CPR group. These findings suggest that CPR surgery induces plastic changes in some neurons of the insular cortex and that PSA-NCAM activation enhances synaptic response to induce central sensitization.

V. CONCLUSION

In the present study, I developed a novel animal model of pain by inducing central sensitization in rats. I show that our new model can be used to investigate the mechanism of central sensitization, which have previously been associated with pain symptoms.[29] Moreover, the CPR model only requires a short time period to induce pain. Nonetheless, further studies are required to elucidate the mechanisms underlying the induction of neuroplasticity and the reconstruction of neural circuits. I also expect that the modulation of neural circuitry induced by our CPR model can be applied to the investigation of many other diseases associated with abnormal connectivity synchronization. As the modeling procedures were complex and more than 3 hr was required to perform all steps, I utilized a single electrical stimulation parameter in our experimental protocol. However, optimization of the stimulation parameter may reduce the stimulation time and increase the efficacy of inducing neuropathic pain induction.

My empirical data suggest that our new CPR pain modeling, which requires short experiment period, can induce the mechanical allodynia with neuroplastic changes in insular cortex. This new pain modeling may contribute to further studies for investigating the role of neuroplastic changes in central sensitization pain inducing mechanisms.

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ABSTRACT (IN KOREAN)

뇌 섬엽 영역의 신경 가소성 변화를 통한 새로운 신경병증성 통증 모델 형성

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윤 민 식

신경병증성 통증은 중추 신경계의 비정상적인 민감도 증가로 인해 발생하게 된다. 아직 민감도가 증가하는 정확한 발생 기작에 대해서는 더 많은 연구가 필요한 상황이나, 최근 연구 결과에 따르면 통증 관련 회로의 가소성의 변화가 고도의 민감화를 유도하게 되고 그 결과 신경병증성 통증을 유발하는 하나의 원인이 될 수 있다는 사실이 밝혀졌다. 그러나 현재 이용되고 있는 동물 통증 모델로는 가소성 변화에 의한 민감화 기작에 대한 연구를 진행하는 대에 어려움이 있다. 따라서 본 연구를 통해 신경회로 재구성 방법인 CPR 수술법을 이용한 새로운 동물 통증 모델을 제시하고 이를 통해 신경가소성에 의한 통증 기작에 대한 연구의 가능성을 제시하고자 한다.

NMDA 와 Ro 25-6981를 쥐 뇌의 섬엽 위치에 주입하고 난 후 왼쪽 발바닥 가장자리 부위에 전극을 삽입하여 전기자극을 전달함으로써 통증

관련 신경 회로를 재구성 하며, 본 프레이 검정 방법을 통해 실제 쥐에서 이질통이 발현 되었는지를 확인한다. 더 나아가 발생된 이질통이 뇌 섬엽 부위에서의 가소성의 변화에 기인한 것인지를 확인하기 위해 PSA-NCAM 이라는 신경세포 결합단백질의 면역 형광을 이용하였다.

통증 관련 회로의 CPR 모델링을 진행한 결과 수술 1 일 후부터 민감도가 낮아지는 것을 확인하였으며 21 일 동안 민감도가 유지 되는 것을 확인하였다. 또한 전기자극만을 준 mod-CPR 1 그룹과, NMDA 와 Ro 25-6981 만을 주입한 mod-CPR2 그룹과 비교하였을 때, 수술 전에 비해 감소한 회피 반응 역치 값의 감소 비율은 CPR 그룹에서 유의미한 차이로 높은 것을 확인하였다. (CPR: 91.68 ± 1.8%, mod-CPR 1: 42.71 ± 3.4%, mod-CPR 2: 18.34 ± 5.1% $p < 0.001$) 이를 통해 CPR 수술법에서만 이질통이 유발 되는 것을 확인 할 수 있었으며, 더 나아가 면역 형광 방법을 통해 CPR 그룹에서만 PSA-NCAM 이 염색되는 것을 확인함으로써, 섬엽 영역에서의 신경 가소성이 민감화에 원인이 된다는 것을 알아내었다.

우리가 제시한 실험결과를 바탕으로 CPR 수술이 뇌 섬엽 부위의 가소성 변화를 유도하고 통증 관련 신경 회로를 변형시켜 중추 신경계의 민감화를 유도함으로써 신경병증성 통증을 유발한다는 것을 확인하였다.

핵심 되는 말: 신경병증성 통증, 뇌 섬엽 피질, 신경 가소성 변화, 과도한 민감화, 기계적 이질통