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学　位　論　文

Involvement between social defeat stress and pain-related behavior in an application of the nucleus pulpous rat model
（ラット髄核留置モデルにおける疼痛関連行動と社会的ストレスとの関与）

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Involvement between social defeat stress and pain-related behavior in an application of the nucleus pulposus rat model

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ABSTRACT

Study Design: Controlled, interventional animal study.

Objective: To investigate whether or not pain-related behaviors induced by the autologous nucleus pulposus (NP) rat model are prolonged by social defeat stress (SDS). Additionally, this study also evaluated the expression of glial fibrillary acidic protein (GFAP) and ionized calcium-binding adaptor molecule-1 (Iba-1) in the dorsal root ganglion (DRG), spinal cord (SC), and ventrolateral periaqueductal gray matter (VLPAG) using the NP rat model with SDS.

Summary of Background Data: It has been reported that the mechanical withdrawal threshold is reduced by stress. Psychophysical stress affects GFAP protein level in the central nervous system (CNS). GFAP and Iba-1 expression in the DRG and SC play an important role in the neuropathic pain state.

Methods: Adult female Sprague-Dawley (SD) rats were used. Application of SDS to SD rats was achieved through a repeated daily 15 minutes exposure to Long Evans (LE) rats for 8 days. Autologous NP to the left L5 was applied to the DRG of SD rats. Mechanical withdrawal thresholds were measured for 35 days. Plasma corticosterone concentrations were measured to assess the stress response caused by SDS. Immunohistochemical localizations of GFAP and Iba-1 in the DRG, SC, and VLPAG were performed, and the number of immunoreactive (IR) cells were counted.

Results: There was a decrease in the mechanical withdrawal thresholds in the NP group for 21 days compared with the sham group, whereas there was a significant decrease in the thresholds in the NP+SDS group for 35 days. The concentration of plasma corticosterone increased immediately after SDS. In the NP and the NP+SDS groups, the expression of GFAP and Iba-1 in the DRG and SC significantly increased up to day 21. In the sham+SDS and NP+SDS groups, expression of GFAP in the VLPAG decreased until day 35, while there was no significant change in the Iba-1 expression.

Conclusion: SDS prolongs the pain-related behavior caused by NP. Changes of GFAP

expression in the VLPAG were associated with pain-related behavior of the NP+SDS group during the late phase. These results suggest that psychological chronic stress might delay recovery from pain-related behavior induced by LDH model.

Key words

Lumbar disc herniation, Neuropathic pain, Social defeat stress, periaqueductal gray matter, ionized calcium-binding adaptor molecule-1, glial fibrillary acidic protein

Key points

・We used an NP applied model with social defeat stress (SDS) to investigate the association between stress and pain-related behavior and the change of expression in the dorsal root ganglion (DRG), spinal cord (SC), and ventrolateral periaqueductal gray matter (VLPAG).

・The reduction of thresholds in the NP + SDS group lasted longer than those in the NP group.

・In the NP+SDS group, there was a significant decrease in the number of GFAP-IR cells in the VLPAG compared with the sham group.

INTRODUCTION

Lumbar disc herniation (LDH), which is one of the most common diseases that cause low back pain and radiculopathy, is caused by both mechanical compression and inflammation.[1–10] The severity of LDH symptoms is not correlated with the size of the herniated disc. Several animal models have shown that the expression of proinflammatory cytokines and glial cells in the spinal cord and dorsal root ganglion (DRG) is implicated in neuropathic pain states.[11–14] Therefore, chemical factors have become the focus as pharmacological targets. However, some recommended pharmacological treatments for patients with chronic pain have been limited to pain relief, and chronic conditions remain difficult to treat and prevent. While it is a major concern for medicine and public health to treat chronic pain, in clinical situations, the background of chronic pain patients varies.

Psychological and social factors are believed to be involved in the onset and prolongation of chronic pain.[15,16] but it is difficult to evaluate the impact of stress on the onset and chronicity of pain. In particular, stress and depression are clearly closely involved in chronic low back pain with or without radiculopathy and daily disability, but the mechanisms have yet to be clearly elucidated. One of the mechanisms of chronic pain is thought to be descending inhibitory system dysfunction caused by stress, anxiety, and depression.[17] In our previous study using functional magnetic resonance imaging to investigate brain activation, low back pain patients with psychiatric problems showed dysfunction of the descending inhibitory system, which is associated with central analgesic activity.[18] In addition to the variations in background, cross-sectional observation studies are insufficient to prove causal relationships between psychosocial stress and subsequent low back pain. Furthermore, prospective longitudinal studies with the same degree and frequency of stress intervention among the general population or for low back pain patients would be impossible to perform. Therefore, *in vivo* studies using animal models are appropriate to investigate pain-related behavior, and some targets expressed in the DRG, spinal cord, and brain with or without consistent stress intervention can be detected histologically. Several experimental methods are used to induce physical and psychological stresses, such as chronic stress due to repeated

restraint, forced swims, cold environments, and restraint stress, which can be caused by hyperalgesia.[19–22] In regard to physical stress, we have found that physical restraint stress induces a reduced mechanical withdrawal threshold and delays in the recovery of neuropathic pain induced by the autologous nucleus pulposus (NP) in a rat model, which is the same model used in the present study.[23] However, there are few reports on the impact of social defeat stress (SDS) on pain-related behavior due to neuropathic pain, and the role of psychological stress in lumbar spinal disorders, especially LDH, in rats has not been investigated. SDS is a method used to reflect psychological and social stress[23,24] and induces a depressive condition that is useful to investigate the efficacy of pharmacological treatments for depression.[25] For these reasons, we chose SDS as the psychological stress to investigate its impact on an LDH model, which reflects the most common lumbar spinal disorder in the clinic. When an organism is exposed to stress, information about the stressful situation reaches an array of brain regions, including parts of the limbic system and areas involved in sensory processing. The output from these areas is funneled through the nucleus paraventricularis of the hypothalamus, where it can give rise to the activation of two hormone systems. These systems include the rapid sympatho-adrenomedullar system and the slow-acting hypothalamo-pituitary-adrenal system.[26] Then, adrenaline and corticosterone levels increase due to activation of these systems, which is thought to be a reflection of stress. Since SDS methods vary and are modified between studies, we measured plasma corticosterone after SDS to observe whether the rats received stress. The dorsal horn of the spinal cord and the periaqueductal gray (PAG) are important components of the descending inhibitory system. The functions of PAG include roles in fear, anxiety, defensive reaction and autonomic regulation. It also plays a crucial role in pain modulation.[27,28] Anatomically and functionally, the PAG is divided into four longitudinal parts (the dorsomedial, dorsolateral, lateral, and ventrolateral columns) that run parallel to the aqueduct; the ventrolateral column is also called the ventrolateral periaqueductal gray (VLPAG).[27] Activation of glial cells such as astrocytes and microglia in the spinal cord plays an important role in neuropathic pain, and the VLPAG is also involved in chronic pain.[27]

Ionized calcium-binding adaptor molecule-1 (Iba-1) is a marker of endoneurial macrophages and microglia, and glial fibrillary acidic protein (GFAP) is a marker of satellite glial cells and astrocytes.[29] Chronic stress decreases GFAP level in the cerebral cortex, amygdala, and hippocampus.[30–32] After chronic restraint stress, the expression of GFAP in the VLPAG has been shown to decrease and there are decreases of the mechanical withdrawal thresholds also observed.[28]

The aims of the current study were to assess the effect of the SDS on pain-related behavior induced by NP application in rats, and to determine the changes of GFAP and Iba-1 expression in the VLPAG, DRGs, and spinal cord, with or without SDS.

MATERIALS AND METHODS

Animals

A total of 272 female Sprague-Dawley (SD) rats (8 weeks old, 150-180 g; Japan SLC, Shizuoka, Japan) and 12 male Long Evans (LE) rats (9 weeks old, 325-350 g; Japan SLC) were used. Animals were housed in plastic cages at room temperature (RT; 21-24°C) on a 12-hour light/dark cycle with free access to food and water.

The animal experiments were carried out under the guidance of the Fukushima Medical University Animal Care and Use Committee and the Guidelines for Animal Experiments, and in accordance with the Japanese government's law and fundamental guidelines concerning the protection and control of animals.

Experimental Groups

SD rats were divided into 2 surgical groups: the NP application group (NP group) and the sham operated group (sham group). Naive rats were used for blood sampling.

Surgical Procedure[33–36]

A combination of 0.3 mL of medetomidine hydrochloride (1.0 mg/mL; Nippon Zenyaku Kogyo, Fukushima, Japan), 0.8 mL of midazolam (5.0 mg/mL; Astellas Pharma, Tokyo,

Japan), and 1.0 mL of butorphanol tartrate (5.0 mg/mL; Meiji Seika Pharma, Tokyo, Japan) were prepared as the anesthetic. Animals were anesthetized by an intraperitoneal injection of 0.1 mL/100 g of body weight. Rats were placed in a prone position followed by an incision made in the middle of the spine at the L4–L6 level. The fascia was incised along the left side of the supraspinous ligament for approximately 20 mm. The multifidus muscles were moved laterally in order to expose the facet joint between the left 5th and 6th lumbar vertebrae. Next, left L5/6 facetectomy was then performed. After exposing the left L5 spinal nerve and DRG, NP harvested from the tail was applied to the left L5 DRG (NP group). In the sham group, the same surgical procedure was performed except for the lack of the application of NP to the DRG.

Social defeat stress protocol[37,38]

The paradigm for the social defeat stress model consists of placing a smaller experimental rat (intruder) in the home cage of a larger and aggressive LE rat, which will then defend its territory and defeat its intruder.[23] SD female rats and LE male rats were housed in 2 separate cages. A SD female rat was subsequently placed as an intruder in the resident male's cage for 15 minutes per day starting from 4 days preoperative to 4 days postoperative. If the fighting became too intense during the SDS, or there was a potential sexual interaction between the intruder and resident rat, the intruder rat was transferred to a protective plastic cage within the resident's home cage, which allowed for intense visual, auditory, and olfactory interactions.[37,38] This cage was large enough for intruders to move freely. SDS was performed for 15 minutes between 9:00~12:00.

Measurement of Plasma Corticosterone Concentrations

Radioimmunoassay (RIA) was used to compare the concentrations of plasma corticosterone between the naive rats with (SDS group) or without (naive group) SDS (n=6 in each). Plasma samples were collected at 14:00–15:00 due to consideration of the influence of the circadian rhythm. Rats were deeply anesthetized using isoflurane (Wako Pure Chemical

Industries, Osaka, Japan). Blood samples (3.0 mL) were collected from the right atrium using a 23G medical needle and mixed in a test tube with ethylenediaminetetraacetic acid dipotassium salt dehydrate (EDTA-2K). To extract the plasma, samples were centrifuged at 3,000 g for 10 minutes at RT. Supernatants were stored at -20°C with the concentrations of plasma corticosterone measured by the LSI Medicine Corporation. Blood samples in the SDS group were obtained within thirty minutes after the SDS procedure.

Measurement of Mechanical Withdrawal Thresholds[39]

Behavior testing was performed in the 5 experimental groups: NP group, NP + SDS group, sham-operated group (sham group), sham + SDS group, and naive + SDS (n=12 in each group). Sensitivity to non-noxious mechanical stimuli was tested using the von Frey test. Baseline testing was performed at 1 day before SDS to determine the rat's normal responses at baseline. The left hind paw withdrawal responses to the von Frey filament (monofilament sensory tester, Sakai Med, Tokyo, Japan) stimulation of the plantar surface of the footpads was determined at postoperative days 2, 7, 14, 21, 28, and 35. Each rat was placed individually in an acrylic cage with a mesh floor and allowed to acclimatize until cage exploration and major grooming activities ceased. The left lateral-plantar surface of the operated hind paw was stimulated with nine von Frey filaments (1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10.0, 15.0, and 26.0 g; Sakai Med) projected through the mesh floor. The gram designations of the von Frey filaments were based on the manufacturer's ratings. Stimulation was initiated with the 1.0-g filament and was applied until there was bending of the filament for 3 seconds. If the rat did not withdraw its foot after the stimulation, the next-stiffer filament was tested in the same manner. The response was considered positive if the hind limb was lifted with either licking or shaking of the foot as an escape response

Immunohistochemical Analysis

Immunohistochemical examination was performed in the 4 experimental groups, the NP group, NP + SDS group, sham group, and sham + SDS group (n=5 for each group at each

time point). Rats were anesthetized using isoflurane (Wako Pure Chemical Industries, Osaka, Japan), and then perfused with fresh 4% paraformaldehyde in phosphate buffer (PB: 0.1 mol/L, pH 7.4). The brain was quickly removed, and postfixed with 4% paraformaldehyde in PB for 6–8 h, then cryoprotected for 48 h in 30% sucrose in 0.1 M PB at 4°C. Tissues were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek Japan Co. Ltd., Tokyo, Japan) and then frozen at -80 °C.

Similarly, left L5 DRG and the L5 segment of the SC were removed. L5 DRG and SC were postfixed briefly in 4% paraformaldehyde and embedded in paraffin. 2 sections (6 μm thick) were cut from each DRG and each SC and placed individually on separate slides.

Ventrolateral periaqueductal gray

Coronal sections of the brain (30 μm) were cut 8mm caudal to bregma[40] on a cryostat with the free-floating sections washed in 0.01 M phosphate buffer saline (PBS) 3 times at 15 minutes intervals. After blocking with 0.01 M PBS containing 5% normal swine serum for 30 minutes at RT, mouse anti-GFAP antibody (1:1000; Millipore Corporation, Billerica, MA, USA) and rabbit anti-Iba-1 (1:1000; Wako Pure Chemical Indus- tries, Osaka, Japan) were applied overnight at 4°C in 0.01 M PBS with 0.3% Triton-X-100. After washing with PBS, sections were incubated with goat anti-rabbit IgG (for Iba1) Alexa Fluor 488 (green) fluorescent antibody (1:200; Thermo Fisher Scientific, Waltham, MA, USA) or goat anti-mouse IgG (for GFAP) Alexa 488 (green) fluorescent antibody (1:200; Thermo Fisher Scientific, Waltham, MA, USA) in 0.01 M PBS with 0.3 Triton-X for 2h at RT. After rinsing, sections were put onto gelatin-coated slides and dried overnight at 4°C in the dark. Once dry, the sections were mounted on microscope slides with Vectashield mounting medium containing DAPI (H-1200, Vector, Burlingame, CA, USA). The number of GFAP-immunoreactive (IR) cells and Iba-1 cells were counted in a 490×650 μm area of the VLPAG in 5 sections.[28] The number of each section in five slides was added and averaged.

Spinal cord and dorsal root ganglion

Sections were deparaffinized with xylene and 100% ethanol, and pre-treated with Dako

target retrieval solution (Dako North America, Carpinteria, CA) at 95°C for 20 minutes to enhance immunoreactivity. After blocking with 2% normal goat serum applied for 1 hour at RT, rabbit anti-Iba-1 (1:1000; Wako Pure Chemical Indus- tries, Osaka, Japan) or mouse anti-GFAP antibody (1:1000; Millipore Corporation, Billerica, MA, USA) were applied overnight at 4°C by incubation for 1 hour at RT with goat anti-rabbit IgG (for Iba1) Alexa Fluor 488 (green) fluorescent antibody (1:200; ThermoFisher Scientific, Waltham, MA, USA) or goat antimouse IgG (for GFAP) Alexa 488 (green) fluorescent antibody (1:200; ThermoFisher Scientific, Waltham, MA, USA). After rinsing, the sections were mounted on microscope slides with Vectashield mounting medium containing DAPI (H-1200, Vector). Fluorescent staining was analyzed using a fluorescent microscope (BX53, Olympus, Tokyo, Japan) with a digital camera (DP73, Olympus). Two sections of each DRG were used to determine the numbers of GFAP- IR cells and Iba1-IR cells. The percentage of each type of IR neuron of the total number of neurons was computed for the DRG and averaged.[33,35] The numbers of GFAP-IR cells and Iba-1-IR cells were counted in a 150×150 μm area in 5 sections of the ipsilateral SC dorsal horn. The numbers of each IR cell type in the 5 SC slides were added and averaged.[3541]

Statistical Analysis

Plasma corticosterone levels are reported as the median [interquartile range (IQR)]. Comparisons of the plasma corticosterone levels were assessed with the Mann-Whitney U test. Other measurements are reported as the mean±standard deviation (SD). Statistical differences for the behavior testings and histological findings were analyzed using a Dunnett's test. P values <0.05 were considered significant.

RESULTS

RIA Analysis of Plasma Corticosterone

Concentrations of plasma corticosterone in the SDS group was 549.5 ng/mL [range: 383-638 ng/mL], while they were 209 ng/ml [range: 68.9-323 ng/mL] in the naive group. There was a

significant increase in the levels in the SDS group compared to the naive group ($p<0.05$) (Figure 1).

Behavioral Testing

The mechanical withdrawal thresholds in the NP group were reduced starting from day 7 until day 21 compared to the sham group ($p<0.05$). In the NP+SDS group, the thresholds were significantly reduced starting from day2 to day 35 compared to the sham group ($p<0.05$) (Figure 2). There was a difference between the NP and NP+SDS groups at day 28 and day 35 ($p<0.05$). There were no significant differences for the thresholds between the sham, SDS, and sham + SDS groups.

Immunohistochemical Analysis in DRGs

GFAP expression was localized at the satellite glial cells within the DRG (Figure 3-A). There was a significant increase in the percentage of GFAP-IR cells in the NP+SDS group on days 14, 21, and 35 compared to the sham group ($p<0.05$). In the NP group, the percentage of GFAP-IR cells increased on days 14 and 21 as compared to the sham group ($p<0.05$). There was a significant increase in the percentage of GFAP-IR neurons in the sham+SDS group on days 14 and 35 compared to the sham group ($p<0.05$). Although on day 7 there was an increase in the number of GFAP-IR cells in all of the groups, there was no significant difference compared to the sham group (Figure 3-B).

Iba-1-IR endoneurial macrophages were observed within the DRG (Figure 4-A). In the NP+SDS group, there was a significant increase in the Iba-1-IR cells on days 7 and 21 compared to the sham group ($p<0.05$). In the NP group, there was only an increase of the Iba-1-IR cells on day 7 compared to the sham group (Figure 4-B).

Immunohistochemical Analysis in Spinal Cord

GFAP is the marker for astrocytes, and GFAP expression was observed in the SC (Figure 5-A). In the NP+SDS group, the number of GFAP-IR cells increased significantly from day 14

to 21 compared to the sham group ($p<0.05$) (Figure 5-B).

Iba-1 is the marker for microglia, and Iba-1 expression was observed in the SC (Figure 6-A). There was a significant increase in the number of Iba-1 cells in the NP group starting from day 14 until day 21 compared to the sham group ($p<0.05$). In the NP+SDS group, there was a significant increase in the number of Iba-1 cells starting from day 7 until day 14 compared to the sham group ($p<0.05$) (Figure 6-B).

Immunohistochemical Analysis in the VLPAG

GFAP-IR cells were observed around the aqueduct (Figure 8-A). In the NP group, there was a significant decrease in the number of GFAP-IR cells on days 7 and 28 compared to the sham group ($p<0.05$). In the NP+SDS group, there was a significant decrease in the number of GFAP-IR cells on days 7, 28, and 35 compared to the sham group ($p<0.05$) (Figure 8-B). In the sham + SDS group, there was a significant decrease in the number of GFAP-IR cells starting from day 7 until day 35 compared to the sham group ($p<0.05$). Iba-1-IR cells were observed around the aqueduct (Figure 9. A). There was no significant difference in the number of Iba-1-IR cells among the 4 groups at any of the time points (Figure 9-B).

DISCUSSION

There were two major findings in the present study. First, SDS modified mechanical allodynia after NP application to the DRG. Second, the reduction of the pain threshold was associated with the decrease of GFAP-IR cells in the VLPAG.

Increased plasma concentrations of corticosterone are a reflection of stress.[26,42] In the present study, plasma corticosterone concentrations increased after SDS, showing that SDS in this study served as a psychological stressor on rats. SDS methods are different between studies, and some SDS methods lead to a reduction in the pain threshold in normal rats[28,43–46] However, SDS in this study did not cause any reduction in the pain threshold in either naive or sham rats, while the threshold was reduced in rats with LDH. These results

suggest that the SDS method used in this study is not a strong stressor, but does have an impact on the condition of LDH. Furthermore, the pain threshold in the NP+SDS group was reduced earlier and lasted longer under the stress condition compared to the NP group. These results indicate that SDS prolongs the pain-related behavior induced by NP, which is analogous to clinical situations in which psychological factors are associated with aggravation, chronicity, and disability in lower back pain patients.[47,48]

Activation of spinal cord microglia and astrocytes after peripheral nerve injury or inflammation contributes to a reduction in the pain threshold.[49]

According to our previous studies, NP application to the DRG and a crushed spinal L5 distal nerve result in a large number of GFAP-IR cells and Iba-1-IR cells among satellite cells and macrophages, as well as microglia in the spinal cord, which are related to the chronic change of pain-related behavior.[11,50,51] In the present study, both the NP and the NP+SDS groups exhibited a significant increase in GFAP-IR cells and Iba-1-IR cells in the DRG and SC for 21 days. There was no significant difference in these cells among the 4 groups at day 7. These results indicate that there was an influence from the surgical procedure until day 7, and the SDS effect was masked by nerve inflammation from the NP. Therefore, changes in GFAP and Iba-1 expression of the SC and DRG in NP rats with or without SDS are related to the early phase of pain-related behavior due to the application of NP.

On the other hand, the pain-related behavior in the NP group improved from day 28, while that in the NP+SDS group lasted longer than 35 days. And GFAP-IR cells expressed in the VLPAG in the NP+SDS and sham + SDS groups slowly decreased at days 28 and 35. Therefore, astrocytes play a key role in chronic pain-related behavior.

Astrocytes organize not only intercellular communications with surrounding neurons, but also widespread networks via gap junctions in the nervous system.[52,53] Astrocytes sense the same synaptic inputs as neurons and elevate the intracellular calcium. Astrocyte calcium signaling triggers the release of gliotransmitters, such as glutamate, ATP and D-serine. These transmitters modulate presynaptic neurotransmitter release and can affect neuronal activity and function.[54] The glial glutamate transporters expressed in the PAG seem to be

EAAT2,[55] which is only located in astrocytes.[56] EAAT2 is essential for maintaining low extracellular glutamate concentrations and for preventing glutamate neurotoxicity.[57] A loss of GFAP leads to a disturbance in the transfer of EAAT2 from the intracellular space to cell surface.[57] Moreover, psychophysical stress affects the GFAP protein levels in the central nervous system (CNS). It has been shown that chronic psychosocial and unpredictable mild stress can cause a decrease of the GFAP protein levels in the hippocampus.[3158] Depression model rats show reduced GFAP protein levels in the prefrontal cortex, amygdala, and hippocampus as compared to SD rat.[30] Accordingly, SDS in this study affected the decrease of GFAP expression in the VLPAG and the reduction of pain-related behavior at the late phase. Furthermore, glucocorticoids are known to regulate the production of GFAP.[58] Corticosterone treatment in adult rats also decreases GFAP protein levels in several brain regions, whereas adrenalectomy increases GFAP protein levels.[59] In the present study, the plasma corticosterone level increased and GFAP-IR cells decreased in the VLPAG due to SDS. These results imply that the increase in plasma corticosterone level is possibily the cause of the GFAP decrease in the VLPAG, with the decreased GFAP then triggering a reduction of EAAT2 in the VLPAG. Thus, SDS might cause dysfunction of the VLPAG, which is a part of the descending pain modulation system.

Moreover, microglia also play an important role in neuropathic pain. Depressed and schizophrenic patients who have committed suicide show increases in microglia in the prefrontal and anterior cingulate cortices.[60] Animal models of depression, chronic restraint, and social defeat stresses have increased microglia in the prefrontal cortex, amygdala, and hippocampus.[61,62] However, there is no significant difference in the protein levels of CD11b, a microglia marker, in the rostral ventromedial medulla between control and chronic stress groups.[43] In addition, there have only been a few studies that have examined Iba-1 in the PAG after chronic stress. In the present study, we found that there was no significant difference in Iba-1 cells in the VLPAG among the 4 groups. The changes among microglia in the present and previous studies might differ according to variations in psychophysical stress and the phase of investigation.

Study Limitations

Firstly, other brain regions which are associated with the component of the descending inhibitory system were not examined. Secondly, animal species- and sex-related influences on pain-related behavior and substances were not studied. In addition, other setting of SDS such as period of stress, frequency of stress, difference combination of sex for stressor/intruder animals, and other stress methods were not studied. Finally, the role of EAAT2 did not investigate.

Conclusion

In the present study, pain-related behavior induced by NP application was significantly prolonged by SDS. In the early phase, satellite cells and macrophages in the DRG and spinal cord microglia and astrocytes increased by application of NP with/without SDS. In the late phase, astrocytes expression in VLPAG due to SDS decreased and this change was linked with the chronicity of pain-related behavior. These results indicate that psychological chronic stress delays recovery from pain-related behavior induced by LDH model.

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Figure Legends

Figure 1. Plasma corticosterone concentrations. In the SDS group, plasma corticosterone was significantly increased compared to the naive group ($p<0.05$). The median scores and inter-quartile ranges are marked by parallel lines inside the box. The highest and lowest point are indicated as whiskers.

Figure 2. Changes in the mechanical withdrawal threshold of the footpad using von Frey filaments. Withdrawal thresholds in the NP group were significantly reduced starting from 7 to 21 days postsurgery compared to the sham group ($p<0.05$), whereas the thresholds in the NP+SDS group showed significant reductions starting from 2 to 35 days postsurgery compared to the sham group ($p<0.05$). Data were mean ±SD. (n=12 for each group)

Figure 3. Immunohistochemical analysis of GFAP and the percentage of GFAP-IR cells in the DRG. GFAP-IR satellite glial cells were observed within the DRG. (A) In the NP and the NP+SDS groups, the percentage of GFAP-IR cells increased from day 14 to 21 compared to the sham group ($p<0.05$). The percentage of GFAP-IR cells in the NP+SDS and sham+SDS groups decreased starting from day 28. (B) Data were mean ±SD. (n=6 for each group)

Figure 4. Immunohistochemical analysis of Iba-1 and the percentage of Iba-1-IR cells in the DRG. In the NP and the NP+SDS groups, the percent of Iba-1-IR cells increased on days 7 and 21 compared to the sham group ($p<0.05$). (A, B) Data were mean ±SD. (n=6 for each group)

Figure 5. Immunohistochemical analysis of GFAP and the number of GFAP-IR cells in the SC. In the NP+SDS group, the number of GFAP-IR cells increased from day 14 to 21 compared to the sham group ($p<0.05$). (A, B) Data were mean ±SD. (n=6 for each group)

Figure 6. Immunohistochemical analysis of Iba-1 and the number of Iba-1-IR cells in the SC.
In the NP group and the NP+SDS groups, the number of Iba-1-IR cells increased starting from day 7 to 21 compared to the sham group ($p<0.05$). (A, B) Data were mean ±SD. (n=6 for each group)

Figure 7. In the VLPAG, GFAP-IR cells were observed around the aqueduct.
In the sham+SDS and the NP+SDS groups, the number of GFAP-IR cells decreased significantly up to day 35 compared to the sham group ($P<0.05$). (A, B) Data were mean ±SD. (n=6 for each group) ＊VLPAG: ventrolateral periaqueductal gray ＊＊LPAG: lateral periaqueductal gray ＊＊＊ DLPAG: dorsolateral periaqueductal gray ＊＊＊＊ DMPAG: dorsomedial periaqueductal gray

Figure 8. In the VLPAG, Iba-1-IR cells were observed around the aqueduct.
The number of Iba-1-IR cells showed no significant differences among the groups at each of the time points. (A, B) Data were mean ±SD. (n=6 for each group)
＊VLPAG: ventrolateral periaqueductal gray ＊＊LPAG: lateral periaqueductal gray ＊＊＊ DLPAG: dorsolateral periaqueductal gray ＊＊＊＊ DMPAG: dorsomedial periaqueductal gray

Figure 1

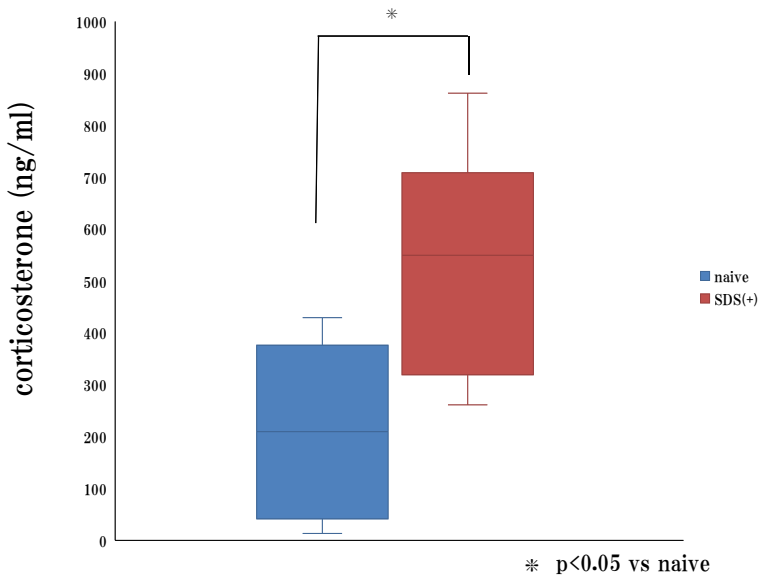


Figure 2

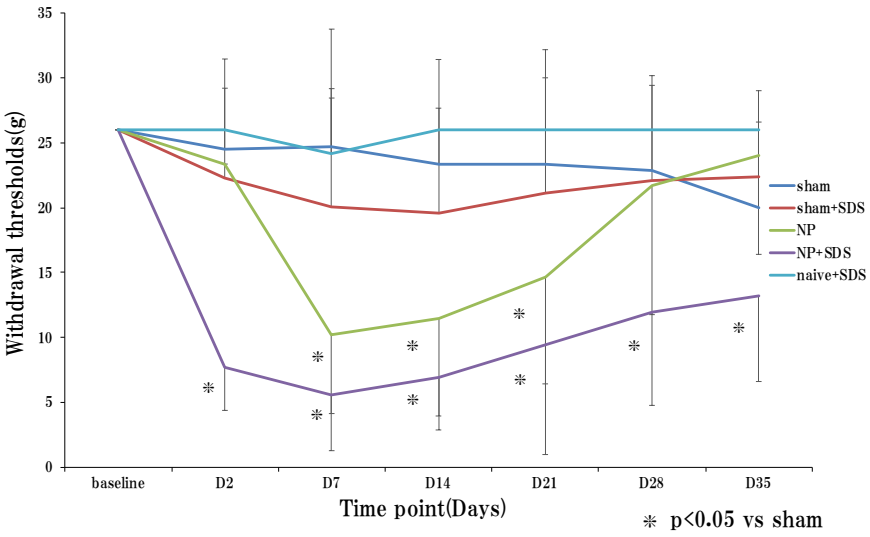
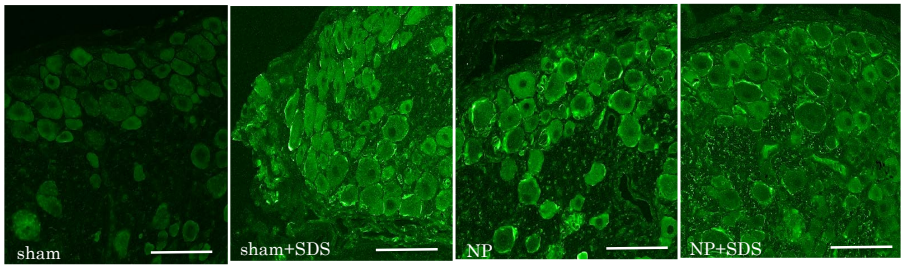


Figure 3-A



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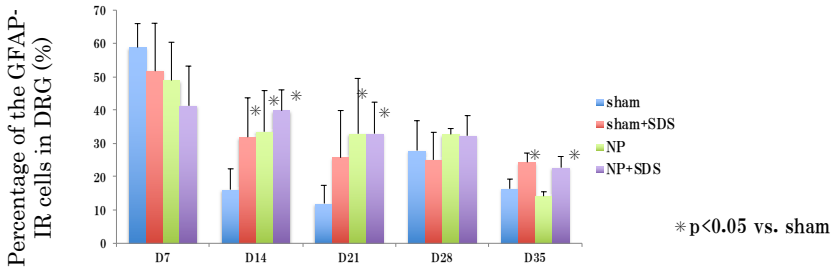
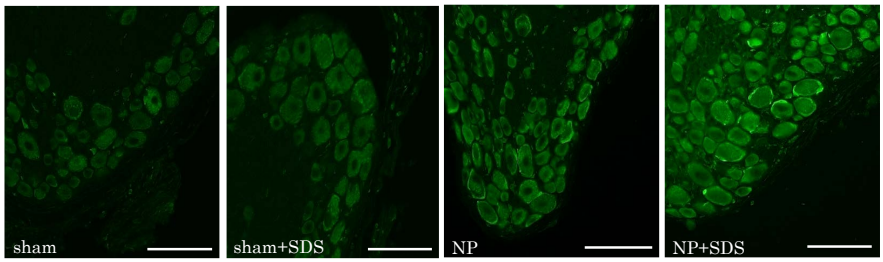


＊p<0.05 vs. sham

Figure 4-A



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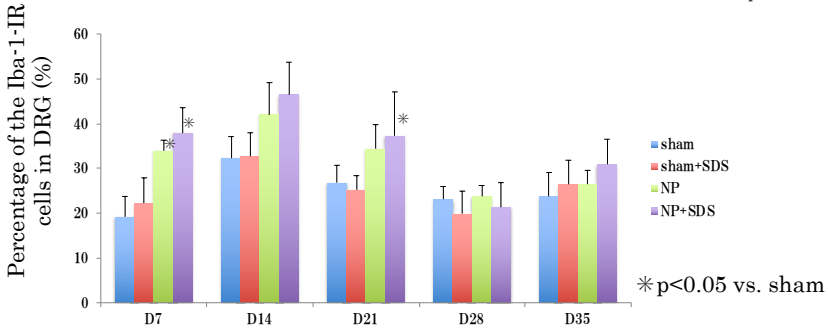

＊p<0.05 vs. sham

Figure 5-A

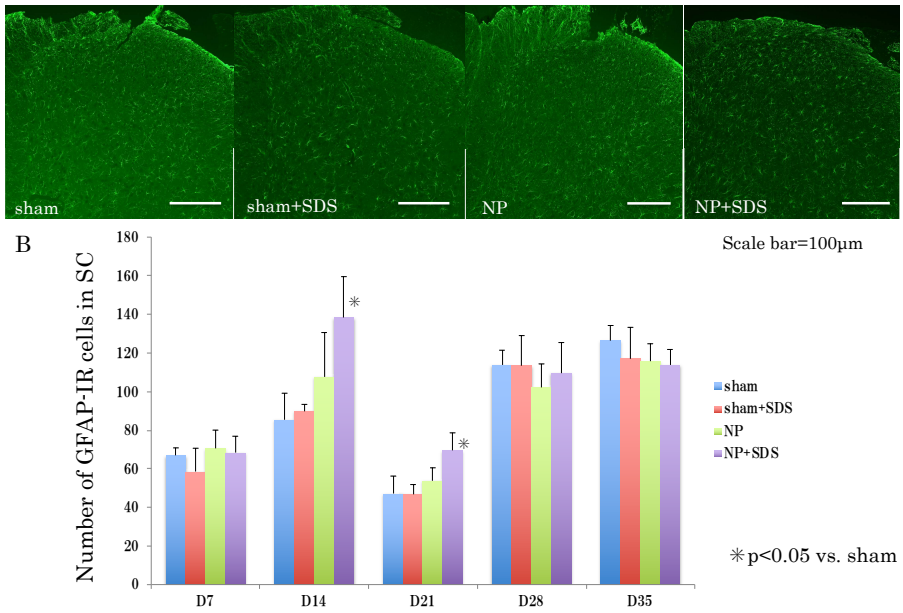

Figure 6-A

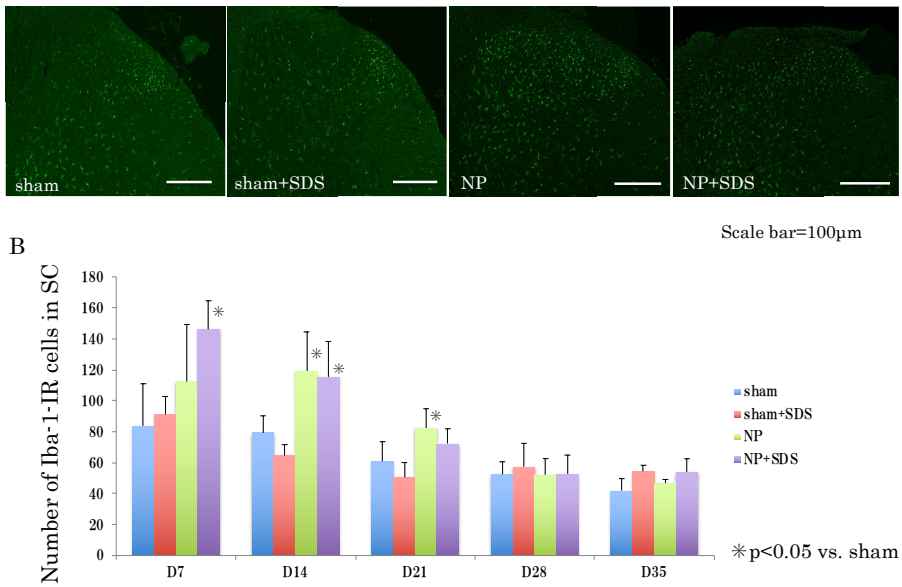

Figure 7-A

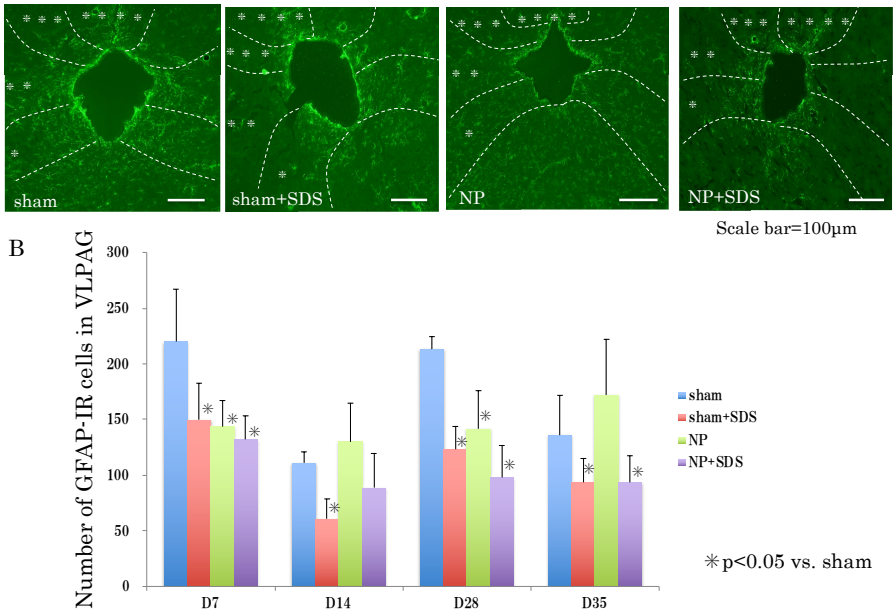
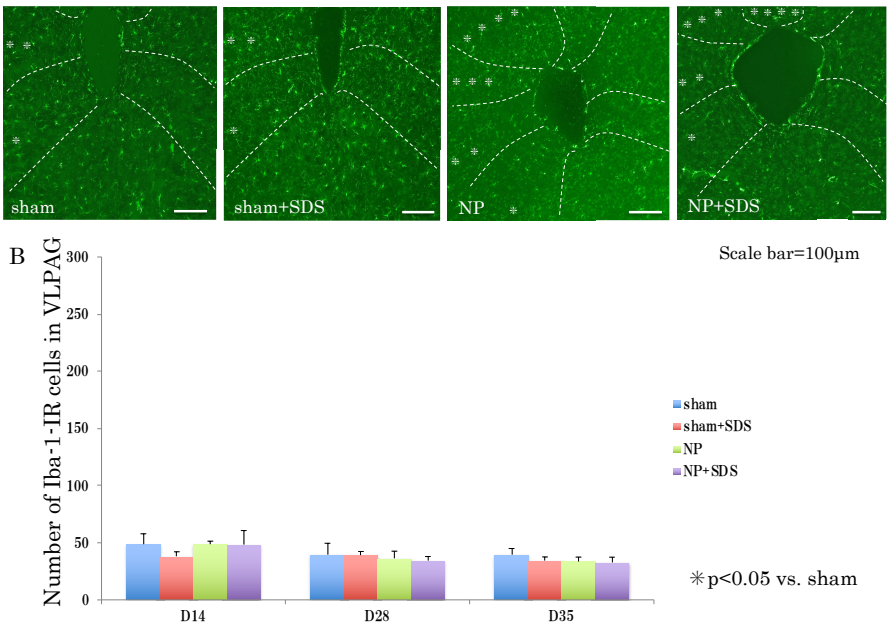
Figure 8-A