Introduction

Lumbar disc herniation (LDH) commonly causes low back pain and neuropathic pain, which is characterized by persistent pain, hyperalgesia and allodynia.[1,2] These symptoms are induced by nucleus pulposus (NP) herniated from the lumbar vertebral disc in both a mechanical and inflammatory manner.[3-11] Several studies have demonstrated that various proinflammatory cytokines,[3,4,10,12,13] monoamine-derived substances[12,14-16] and other factors[17-19] contribute to the pathogenesis of inflammation and neuropathic pain in the state of LDH.

Opioid drugs mainly produce analgesia through activation of the μ-opioid receptors (MORs).[20,21] MORs are widely expressed in the peripheral and central nervous systems: several nuclei of the brain—i.e. Caudate putamen (CPu), nucleus accumbens (NAc), periaqueductal grey matter (PAG), rostral ventromedial medulla and so on—as well as the spinal cord (SC), dorsal root ganglions (DRGs) and peripheral tissues.[22-25] In inflammatory pain rodent models, the expression of MOR mRNA and/or protein increase in both the SC and DRG, amplifying the analgesic potency of MOR agonists.[21,26-28] In most neuropathic pain models, on the other hand, the expression of MOR mRNA and/or protein on the injured side decreases; therefore, the analgesic potency of MOR agonists is attenuated.[25,29-33] In the brain, the CPu expresses MORs in both patches and matrix compartments and is thought to be important for pain modulation.[34,35] The NAc is known as a component of the mesolimbic dopamine

system. MOR levels in the NAc have been thought to be important for pain modulation.[33,36-38] In addition, the PAG is the original nucleus of descending pain modulatory system, and activation of MORs within the PAG results in potent analgesia.[39,40] The expression of MORs has been suggested to vary according to the pathophysiological condition, time course and location, and the relationship to neuropathic pain is not completely understood.

The purpose of the present study was to demonstrate the relationship between dynamic temporal and spatial changes of MOR expressions and pain-related behavior using a rat lumbar disc herniation model.

MATERIALS AND METHODS

Animals

A total of 91 adult female Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) initially weighing 190–210g were used in this study. During the experiments, the rats were housed in plastic cages with woodchip bedding at room temperature (21–24 ℃) in a 12-h light/12-h dark cycle. Water and food were available *ad libitum*.

Animal experiments were carried out under the supervision of the Animal Care and Use Committee in accordance with the Guidelines for Animal Experiments of Fukushima Medical

University and the Japanese Government Law Concerning the Protection and Control of Animals.

Experimental Groups

The rats were divided into two surgical groups: the NP-application group (NP group, n = 43) and the sham-operated group (sham group, n = 37).

Surgical procedure

A mixed anesthetic was prepared with 0.3 mL medetomidine hydrochloride (0.3 mg/kg;), 0.8 mL midazolam (4.0 mg/kg) and 1.0 mL butorphanol tartrate (5.0 mg/kg). Before surgery, The rats were anesthetized by intraperitoneal injection of 0.1 ml/100 g of body weight of mixed anesthetic (0.3 mg medetomidine hydrochloride, 4.0 mg midazolam and 5.0 mg butorphanol tartrate).

The surgery followed a previously described procedure.[11,38,41,42] Briefly, each rat was placed in the prone position, and left L5/6 facetectomy was performed. After the left L5 spinal nerve and DRG were exposed, NP harvested from the tail was applied to the left L5 DRG (NP group). In contrast, no NP was applied in the sham group rats.

Behavioral Testing

Sensitivity to non-noxious mechanical stimuli was tested in a manner similar to the von Frey

test used in previous reports.[11,13,15,38,41-44] The left hind paw withdrawal response to von Frey hair (SAKAImed, Tokyo, Japan) stimulation of the lateral plantar surface of the footpads was investigated at days 0 (baseline), 2, 7, 14, 21 and 28 days after surgery (n=12 in each group). Each rat was placed in an acrylic cage with a mesh floor and allowed to acclimate for at least 20 minutes. The lateral plantar surface of the operated hind paw was stimulated with 9 von Frey filaments (1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10.0, 15.0 and 26.0 g) threaded under the mesh floor. Stimulation was initiated with the 1.0 g filament. The filament was sequentially applied to the paw surface just until the filament bent and was held for approximately 3 seconds. The response was considered positive if the rat lifted the affected limb, coupled with either licking or shaking of the foot as an escape response.

Immunohistochemistry

Immunohistochemical examinations were performed before surgery in the control and 14 days after surgery in the NP group (n = 6, each group). Rats were anesthetized using isoflurane (Wako Pure Chemical Industries, Osaka, Japan), perfused with fresh 4 % paraformaldehyde in phosphate buffer (PB: 0.1 mol/L, pH 7.4). The brain and the L5 segments of the SC were quickly removed, and post-fixed 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. The tissues were embedded in Optimal Cutting Temperature (OCT) compound (Sakura Finetek Japan Co. Ltd., Tokyo, Japan) and frozen at -80 °C. The L5 DRGs were removed

subsequently embedded in paraffin. Two sections (6 µm) were cut from each DRG and placed on separate slides.

Caudate putamen, nucleus accumbens, periaqueductal grey matter and spinal cord

Coronal sections of the brain (30 µm) and transverse sections of the L5 spinal cord (10 µm) were cut on a cryostat and the free-floating sections were washed in 0.01-M phosphate buffer saline (PBS) 3 times at 15-minute intervals. The sections were blocked for 30 minutes at room temperature in 0.01-M PBS containing 5% normal swine serum. Sections were incubated with primary rabbit anti-MOR serum (1:1000; Neuromics, Edina, MN, USA) in 0.01 M PBS plus 0.3% Triton-X-100 overnight at 4℃. After being washed in PBS, sections were incubated with donkey-anti rabbit Alexa Flour 488 fluorescent antibody (green) (1:250; Molecular probes) in 0.01M PBS plus 0.3Tx for 2h at room temperature. After rinsing, sections were put onto gelatin-coated slides and dried overnight at 4℃ in the dark. Once dry, the sections were mounted on microscope slides with VECTASHIELD mounting medium containing DAPI (H-1200, Vector, Burlingame, CA, USA).

Dorsal root ganglion

Sections were deparaffinized with xylene and rehydrated with 100% ethanol, followed by PBS. After that, they were pretreated with Dako Target Retrieval Solution (Dako North America, Carpinteria,

CA, USA) at 97 ℃ for 20 minutes to enhance immunoreactivity. After washing with 0.01-M PBS, the sections were blocked for 1 h at room temperature in 0.01-M PBS containing 2% normal donkey serum. Sections were incubated with primary rabbit anti-MOR serum (1:1000; Neuromics, Edina, MN, USA) in 0.01-M PBS plus 0.3% Triton-X-100 overnight at 4℃. After being washed in PBS, sections were incubated with donkey-anti rabbit Alexa Flour 488 fluorescent antibody (green) (1:200; Molecular probes) in 0.01-M PBS containing 2% normal donkey serum for 1 h at room temperature. After being washed with PBS, the sections were mounted on microscope slides with VECTASHIELD mounting medium containing DAPI (H-1200, Vector, Burlingame, CA, USA). Fluorescent staining was analyzed using a DM6000 FS fluorescent microscope (Leica, Wetzlar, Germany).

Immunoblot analyses

Immunoblot analyses were performed on the day before surgery and on days 2, 7, 14, 21 and 28 after surgery (n=5, for each time point). The rats were rapidly decapitated under anesthesia using isoflurane, and the left L5 DRGs and the left L5 segment of the SCs were quickly removed and all specimens were frozen in liquid nitrogen and stored -80 °C. Simultaneously, the whole brain was removed and sliced at 200- to 300-μm thickness using a vibratome, then three nuclei—CPu, NAc and PAG—were identified and quickly removed under a microscope according to an atlas of the rat

brain[45] and also frozen and stored at -80 °C. All samples were homogenized in ice-cold lysis buffer (#9803; Cell Signaling Technology, Danvers, MA, USA), adding 10 μg/ml of leupeptin, 10 μg/ml of aprotinin, 10 μg/ml of trypsin inhibitor and 10 μg/ml phenylmethane sulfonyl fluoride. The protein concentration of each sample was measured using the BCA protein assay kit (Pierce, Rockford, IL, USA). Samples were run on 10 % tris-glycine-SDS buffer for electrophoresis gel (Wako Pure Chemical Industries, Osaka, Japan) for 90 minutes at 100 V and then transferred to polyvinylidene difluoride filter membranes (EMD Millipore Corporation, Billerica, MA, USA) for 3 h at 0.06 A. The membranes were blocked for 1 hour in 10% non-fat milk in tris-buffer saline plus 0.1% Tween-20 (TBST) at room temperature. Then the membranes were washed with TBST, and incubated overnight with diluted primary antibody in 5% bovine albumin in TBST at 4 °C. After washing with TBST, the membranes were incubated with secondary antibody conjugated to horseradish peroxidase (HRP) for 1 h at room temperature. The following primary and secondary antibodies were used: rabbit anti-MOR1 (1:1500; RA10104, Neuromics, MN, USA), goat anti-rabbit IgG HRP (1:5000; Santa Cruz Biotechnology, Dallas, TX, USA); mouse anti-β-actin (1:20000; SIGMA-ALDRICH, St. Louis, MO, USA), and goat anti-mouse IgG HRP (1:10000; Santa Cruz Biotechnology, Dallas, TX, USA).

Positive bands were detected using an enhanced chemiluminescence system (ImageQuant LAS 4000, GE Healthcare UK Ltd, Buckinghamshire, England). Signal intensity from positive bands was

calculated relative to the signal from the internal controls (β-actin-positive bands) using an imaging analysis system (ImageQuant TL, GE Healthcare UK Ltd, Buckinghamshire, England). The ratio in the naive group rats was set as 1.

Statistical Analysis

All values are reported as means ± standard deviations (SDs). Statistical analyses were assessed with the wilcoxon test. P values < 0.05 were considered significant.

Results

Behavioral Testing

From days 2 to 28, the mechanical withdrawal thresholds of the left hindpaw were significantly lower in the NP group than in the sham group ($P < 0.05$). (Fig. 1).

MOR expression in the DRG

MOR immunoreactive (IR) cells were mainly observed in small DRG neurons and the number of MOR-IR cells decreased in the NP group at day 14 (Fig. 2A, B). MOR-positive bands derived from DRGs were detected at 53 kDa (Fig 2C). In the NP group, MOR expression in the DRG

decreased from day 2. On days 7 and 14, MOR expressions were significantly lower in the NP group than in the sham group ($p < 0.05$).

MOR expression in the SC

MOR mainly expressed in the superficial dorsal horn (Fig. 3A,B). In the NP group, MOR expression in the injured side tended to be lower than the contralateral side (Fig. 3B). In the NP group, MOR expression in the left L5 SC began to show a decrease compared to the sham group at day 2. On days 7 and 14, MOR expressions were significantly lower in the NP group than in the sham group ($P < 0.05$).

MOR expression in the caudate putamen (CPu)

MOR-IR cells were strongly expressed in patches (Fig. 4A). The expression levels of MOR in both groups showed no significant differences at each time point (Fig. 4B).

MOR expression in the nucleus accumbens (NAc)

MOR-IR cells were present in both the shell and the core of the NAc (Fig. 5A). At day 2, the expression levels of MOR in the NP group were higher than those in the sham group at days 7 and 14. At day 21, MOR expression in the NP group was significantly lower than that in the sham group

163 ($p < 0.05$) (Fig. 5B).

164

165 **MOR expression in the periaqueductal gray mater (PAG)**

166 In the PAG, MOR-IR cells were observed around the aqueduct (Fig. 6A). The expression levels

167 of MOR in both groups showed no significant differences at each time point (Fig. 6B).

168

169 # Discussion

170

171 In the NP group, during the period of the lower threshold compared to the sham group from

172 days 2 to 14, MOR expressions in both the SC and DRG of the injured side also significantly decreased

173 at days 7 and 14 ($P < 0.05$). In other neuropathic pain rat models, the degree of reduction in the quantity

174 of MORs in the SC and DRG following nerve injury has been reported to correlate with the severity

175 of mechanical allodynia. [23,25,31,33] In the present study, changes of MOR expressions of the left L5 SC

176 and DRG in the NP group might also be related to pain-related behavior in the early phase. These

177 results indicate that the decrease of MOR protein in both the DRG and SC of the injured side might

178 be related to the attenuation of the analgesic potency of MOR agonists in the early phase as previous

179 studies.[25,29-33]

180 MOR has been reported to relate to the generation and severity of mechanical allodynia

following nerve injury.[23,46] MORs are synthesized in DRG neurons and are transported to their central terminals in the superficial dorsal horn and peripheral terminals in peripheral tissues.[47,48] MORs in the SC dorsal horn are expressed in nearly equal amounts on the central terminals of Aδ and C fibers, and on the dorsal horn neurons.[49] In this study, the decrease in MOR expression in the injured-side DRG neurons might contribute to the decrease of MOR expression in the SC. Regarding the NP-applied rat model, previous studies have demonstrated that inflammation is initially induced by proinflammatory cytokines from applied NP in acute phase,[3,4,12,15,50] and then neuropathic pain occurs.[11,42,44] These findings indicate that the expression of MOR may vary according to the pathophysiological condition, and further investigation is needed.

At days 21 and 28, the mechanical threshold was significantly lower in the NP group than in the sham group ($P < 0.05$). The quantity of MOR protein in the DRG and SC, however, showed no significant differences between the two groups. The pathophysiology of neuropathic pain at the late phase could depend on other peripheral and central mechanisms, including location, cytokines, chemokines and other proteins.

In the brain, MOR expressions in the CPu and PAG showed no significant difference between the NP and sham group rats at any time points. The CPu provides a major link between the thalamus and the cerebral cortex, and relates to several functions including pain modulation.[34,51,52] PAG is an original component of the descending modulatory pain system, and MORs in the PAG play a crucial

role in modulating pain. On an immunostaining study using male NP-applied rat model, the number of MOR-IR cells in PAG increased at 7 and 28 days after surgery.[53] In the present study, however, MOR expression increased at day 14 and then decreased. These differences between the previous and the present studies might be influenced by several factors: sex (male vs female), age (9 months old vs 9 weeks old), method (immunohistochemistry vs immunoblotting) and antibody (Biosourse vs Neuromics). The expression and function of MORs in the PAG have been reported to be different for males and females.[40,54] In line with previous studies, our results may indicate that the sex difference of MOR expression in PAG leads to ineffectiveness of MOR agonists in female rats. On the other hand, the threshold and MOR expression in NAc in the NP group at day 21 were significantly lower than those in the sham group. The NAc is known as a component of the mesolimbic dopamine system and plays important roles in pain modulation.[55] In fibromyalgia patients, positron emission tomography has been shown to reduce MOR binding potential in some nuclei including the NAc.[37] In one animal study, intra-NAc administration of an MOR agonist (fentanyl) increased the level of dopamine in the NAc.[56] These facts might indicate that change of MOR expression in the NAc at day 21 might contribute to inducing chronic pain. Since the detailed involvement of MOR expression in the brain is not completely understood, further investigations are necessary.

Limitation

Firstly, the bilateral differences of MOR expression in NAc, SC and DRG were not examined. Secondarily, animal-species-related and sex-related influences on pain and analgesia were not studied. Finally, this rat model does not reflect all pathology of lumbar disc herniation.

Conclusion

In the present study, we first showed changes of MOR expression in the CPu, NAc, PAG, SC and DRG in a rat lumbar disc herniation model. In the early phase, the decrease of MOR protein in both the DRG and SC of the injured side was related to pain-related behavior. In the late phase, the change of MOR expression in the NAc may have been related to prolongation of neuropathic pain.

Figure 1

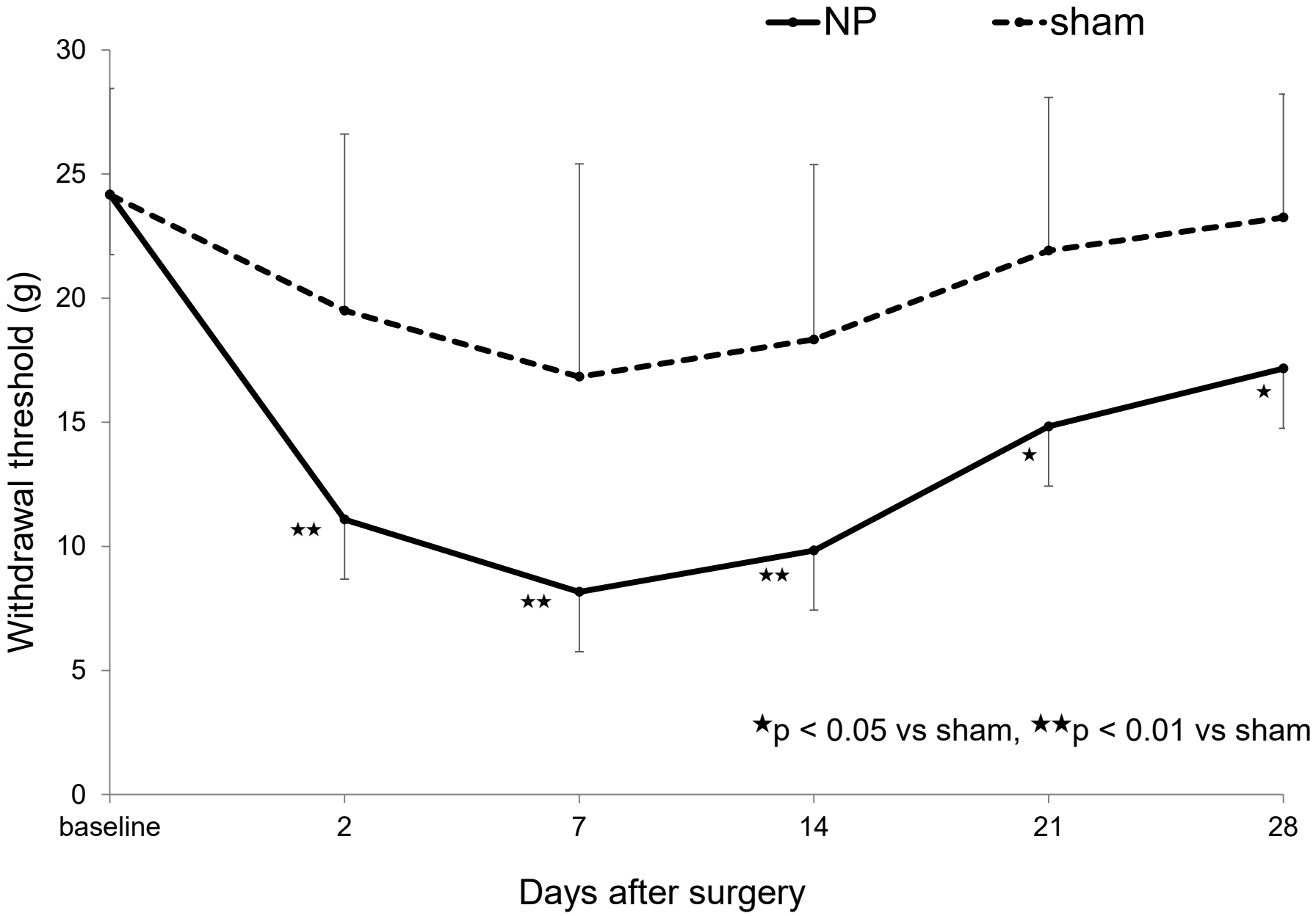
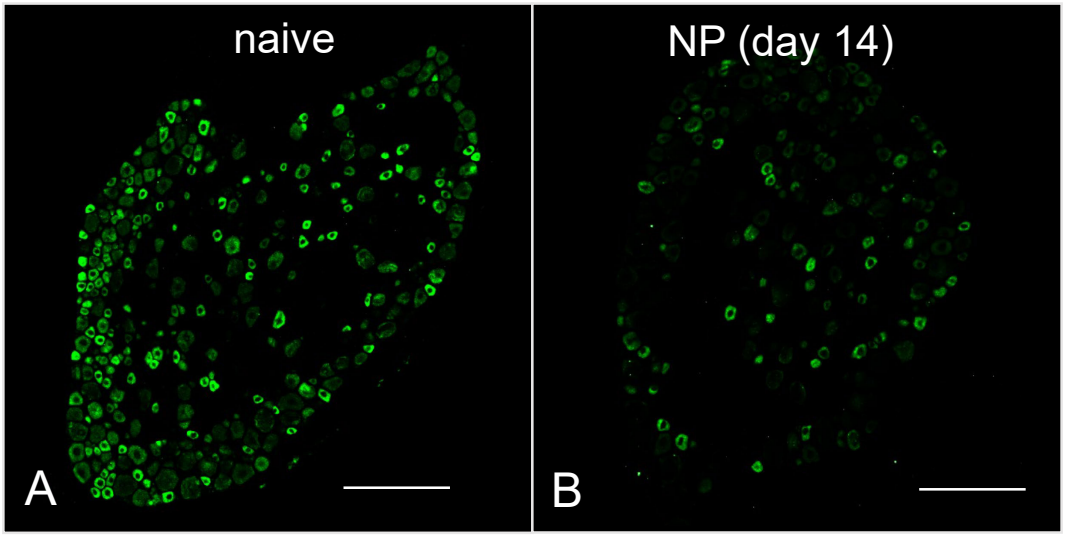
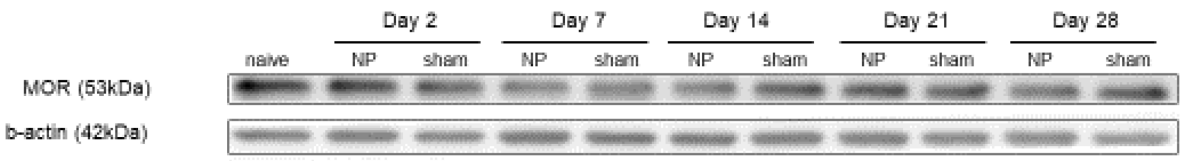
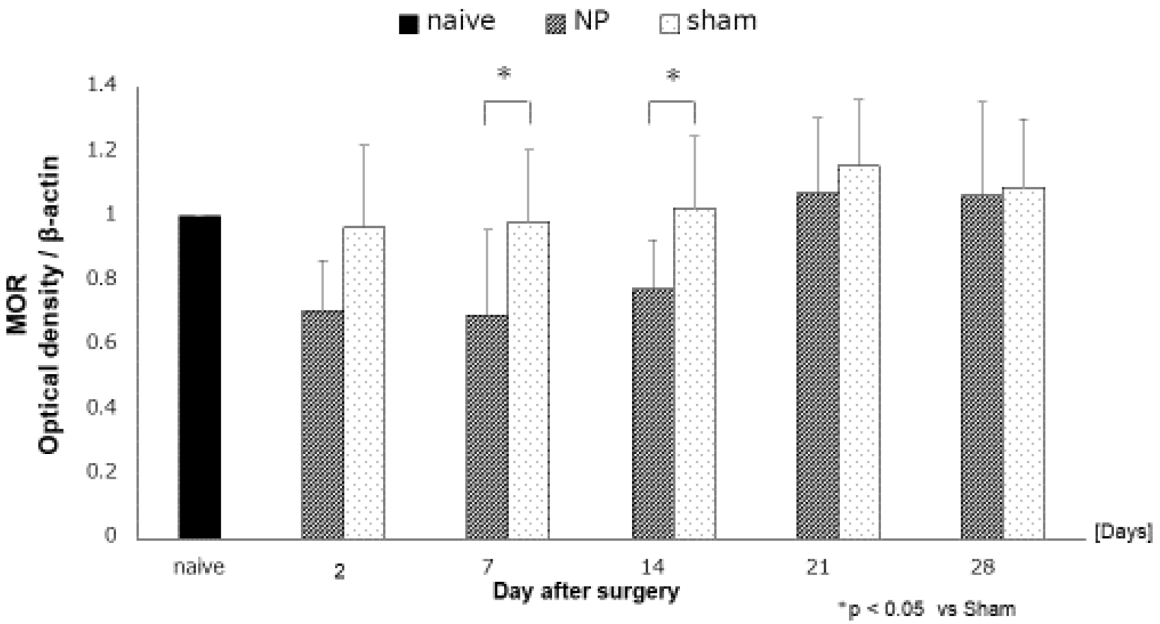

NP
sham
Withdrawal threshold (g)
30
25
20
15
10
5
0
baseline
2
7
14
21
28
Days after surgery
★p < 0.05 vs sham, ★★p < 0.01 vs sham

Figure 2


naive
NP (day 14)
A
B


Day 2
Day 7
Day 14
Day 21
Day 28
naive
NP
sham
MOR (53kDa)
b-actin (42kDa)

C


naive
NP
sham
MOR
Optical density / β-actin
1.4
1.2
1
0.8
0.6
0.4
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*
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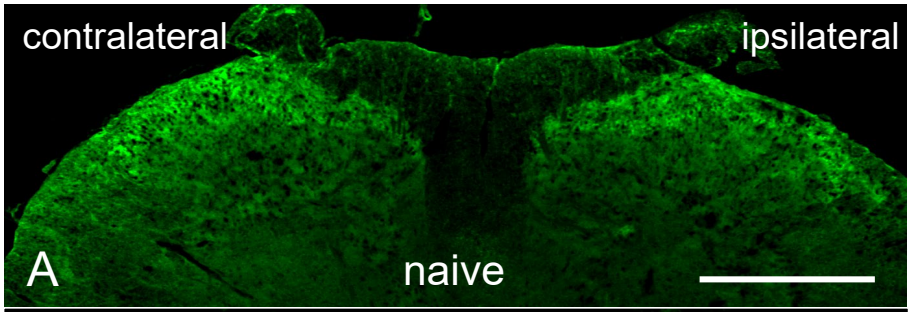
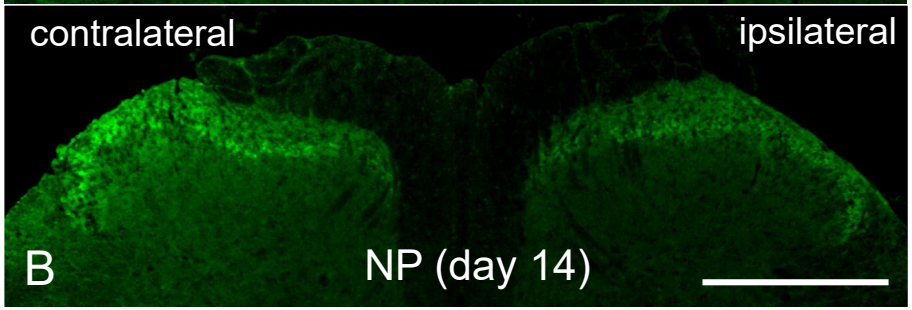
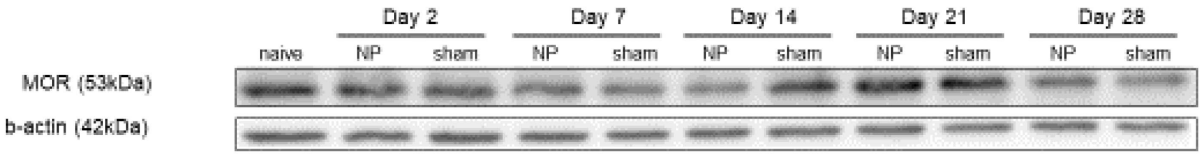
naive
2
7
14
21
28
[Days]
Day after surgery
*p < 0.05 vs Sham

Figure 3


contralateral
ipsilateral
A
naive


contralateral
ipsilateral
B
NP (day 14)


Day 2
Day 7
Day 14
Day 21
Day 28
naive
NP
sham
MOR (53kDa)
b-actin (42kDa)

C

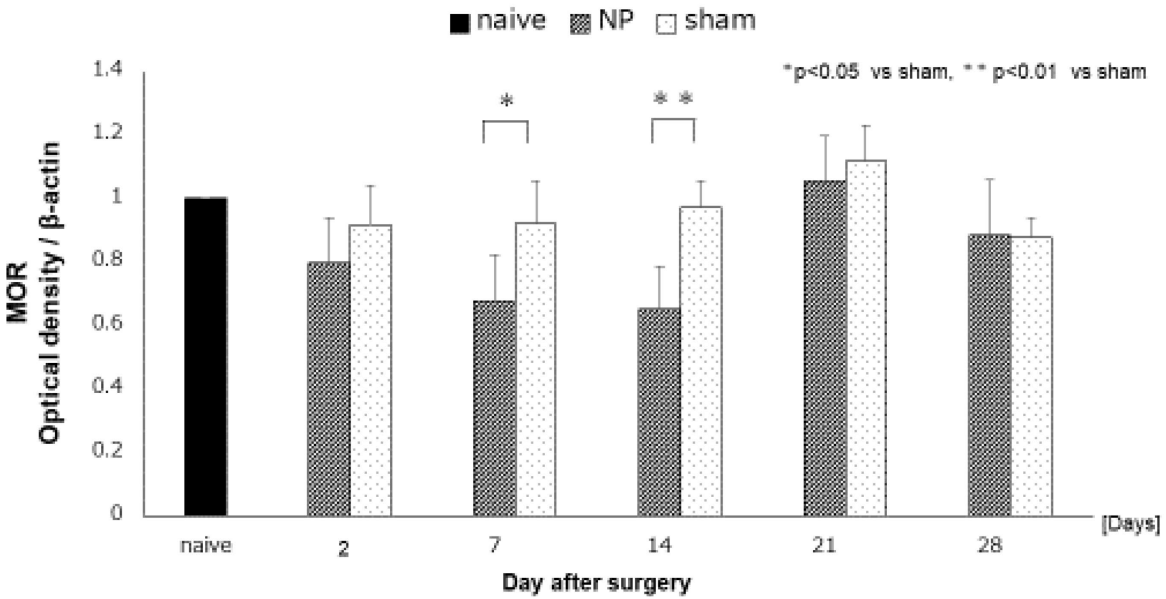
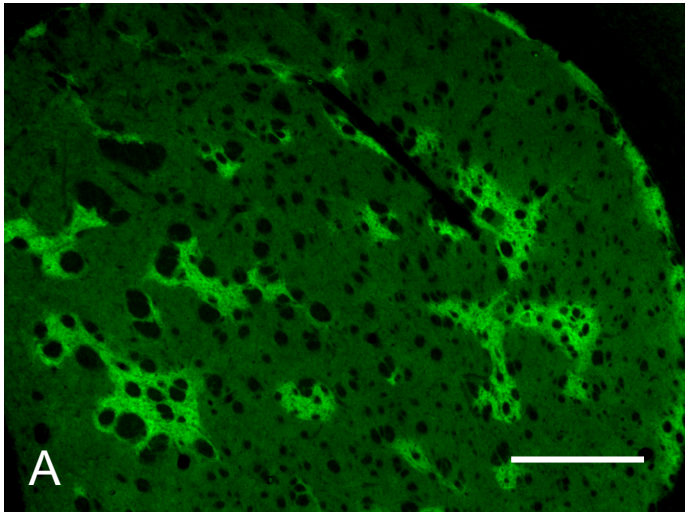
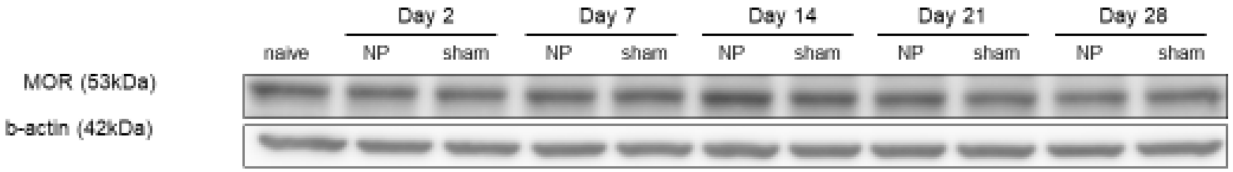

naive
NP
sham
*p<0.05 vs sham, * * p<0.01 vs sham
MOR
Optical density / β-actin
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
* *
naive
2
7
14
21
28
[Days]
Day after surgery

Figure 4


A


Day 2
Day 7
Day 14
Day 21
Day 28
naive
NP
sham
NP
sham
NP
sham
NP
sham
NP
sham
MOR (53kDa)
b-actin (42kDa)

B

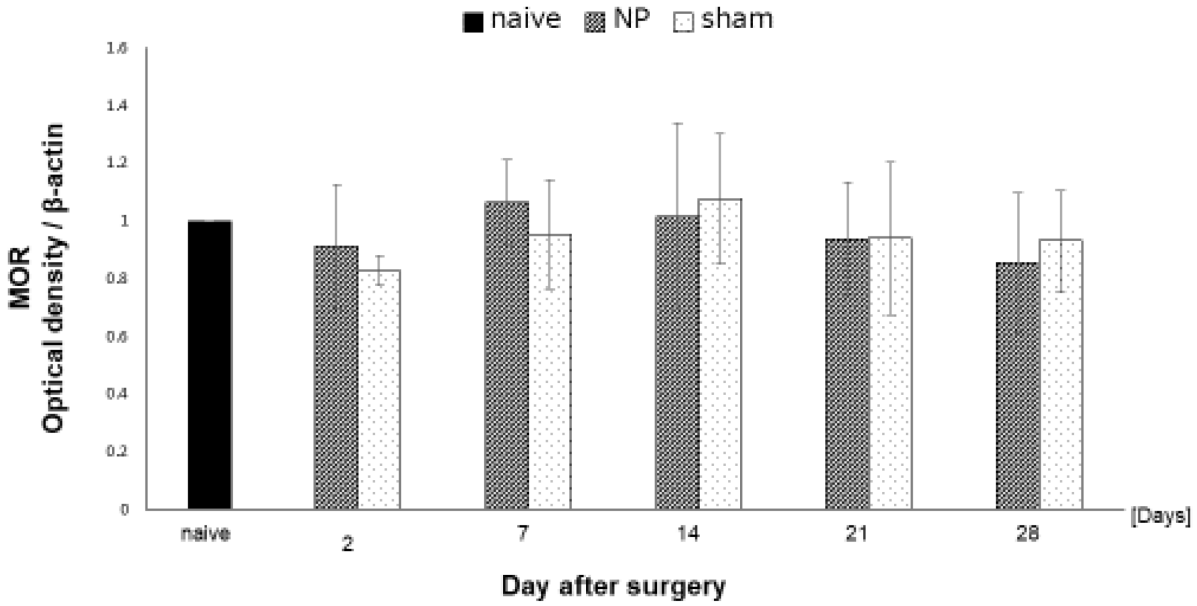
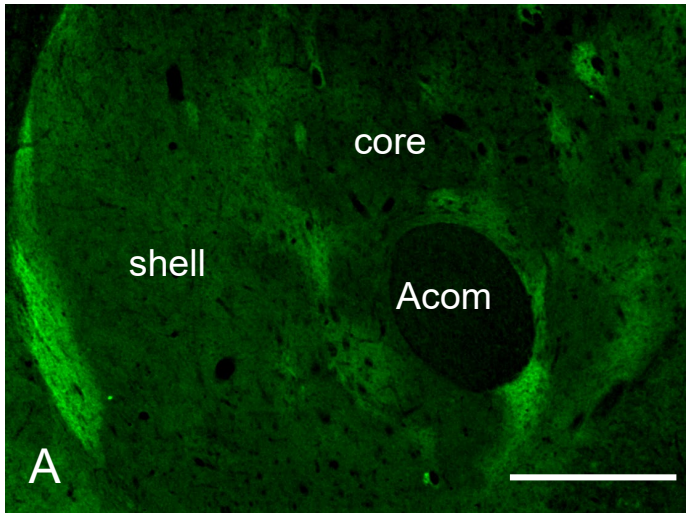
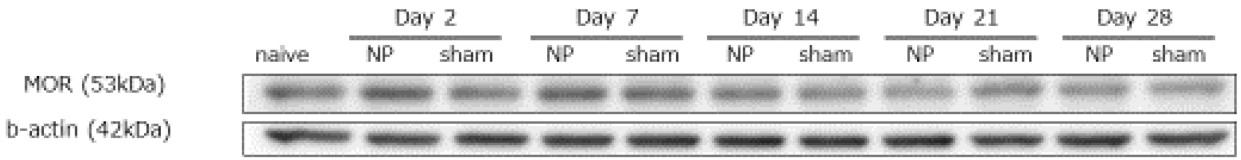
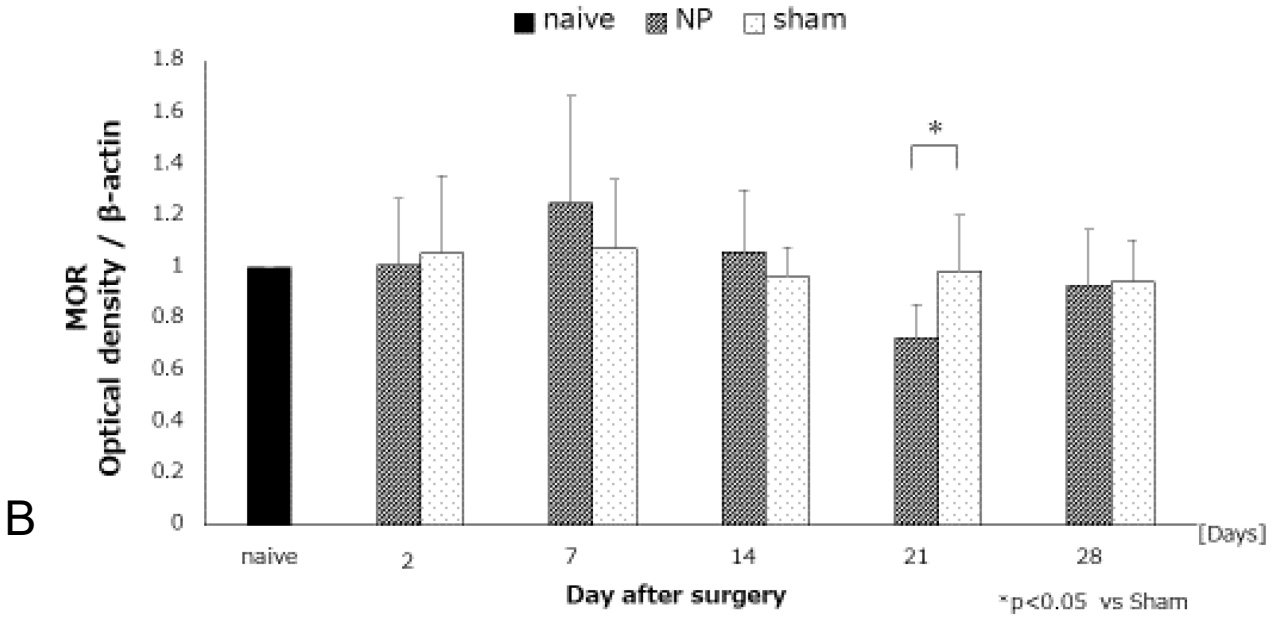

naive
NP
sham
MOR
Optical density / β-actin
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
naive
2
7
14
21
28
[Days]
Day after surgery

Figure 5


core
shell
Acom
A


Day 2
Day 7
Day 14
Day 21
Day 28
naive
NP
sham
NP
sham
NP
sham
NP
sham
NP
sham
MOR (53kDa)
b-actin (42kDa)


naive
NP
sham
MOR
Optical density / β-actin
1.8
1.6
1.4
1.2
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0.8
0.6
0.4
0.2
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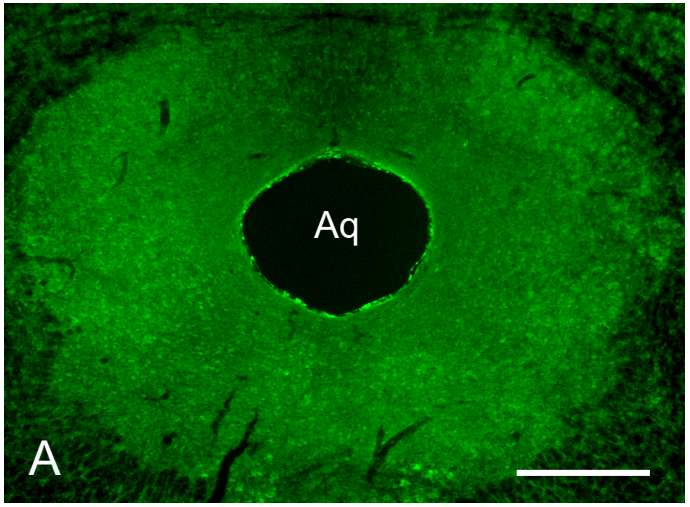
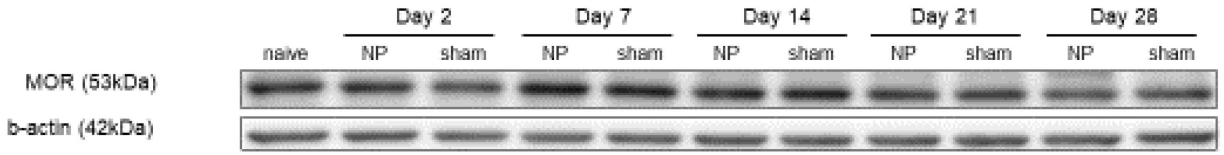
*
naive
2
7
14
21
28
[Days]
Day after surgery
*p<0.05 vs Sham

B

Figure 6


Aq
A


Day 2
Day 7
Day 14
Day 21
Day 28
naive
NP
sham
NP
sham
NP
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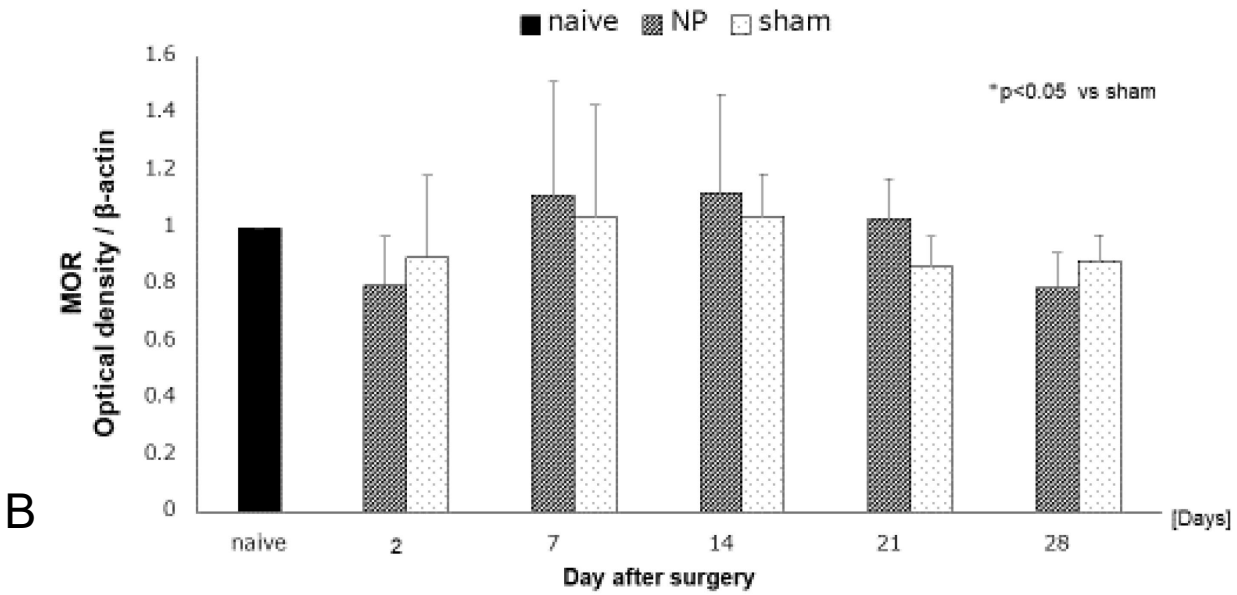
NP
sham
NP
sham
MOR (53kDa)
b-actin (42kDa)


naive
NP
sham
*p<0.05 vs sham
MOR
Optical density / β-actin
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
naive
2
7
14
21
28
[Days]
Day after surgery
B