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Effect of Continuous Passive Motion Initiated After the Onset of Arthritis on Inflammation and Secondary Hyperalgesia in Rats

Running title: The effect of CPM on hyperalgesia in rats

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19 Summary

20 This study investigated the effect of continuous passive motion (CPM) initiated after
21 the onset of arthritis in rats. Rats were injected with 3% kaolin/carrageenan in the
22 knee joint and randomized to the control, immobilization (IM), or CPM group. The
23 knee joints of the IM and CPM groups were immobilized with a cast for 56 days. In the
24 CPM group, CPM exercise was administered for 60 min/day (6 times/week). Joint
25 transverse diameter and pressure pain threshold (PPT) were assessed as indicators of
26 inflammation, and paw withdrawal response (PWR) was assessed as indicator of
27 secondary hyperalgesia. Central sensitization was analyzed by measuring calcitonin
28 gene-related peptide (CGRP) expression levels in the spinal dorsal horn. In the CPM
29 group, the PPT was significantly increased compared with the IM group from 14 to 35
30 days, and PWR was significantly decreased from 14 to 56 days. Additionally, CGRP
31 expression in the super facial layer (I-II) of the spinal dorsal horn (L4-5) in the CPM
32 group was significantly decreased compared with the IM group. Our study found the
33 CPM initiated after the onset of arthritis promoted the recovery of inflammation and
34 mitigated secondary hyperalgesia.

35 Key words

36 Arthritis; Inflammation; Continuous passive motion; Immobilization; Hyperalgesia

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Introduction

Acute pain is associated with inflammation evoked by trauma, surgical procedures, and arthritis. It is ordinarily thought that acute pain is time dependent because inflammation gradually decreases until the growth phase of tissue healing. However, in the case of severe and prolonged tissue damage, the sustained activity of primary afferent fibers induces peripheral sensitization, which increases the efficacy of synaptic transmission between primary afferent fibers and dorsal horn neurons, a process referred to as central sensitization. Severe and long-lasting noxious stimulation from peripheral tissue is a risk factor for chronic pain (Radhakrishnan *et al.* 2003).

Resting the affected side by immobilization using plaster cast or brace is useful for restoring tissue damage and is widely used for medical treatment. However, immobilization can cause muscle weakness (Booth 1977) and joint contracture (Honda *et al.* 2015). Moreover, recent studies have suggested that immobilization may cause hyperalgesia with central sensitization in the spinal cord (Terkelsen *et al.* 2008, Hamaue *et al.* 2013). Terkelsen *et al.* (2008) observed pain induced by transient movement and mechanical hypersensitivity in the distal forearm of human subjects after 4 weeks of cast immobilization. Hamaue *et al.* (2013) demonstrated that cast immobilization produces a time-dependent increase in mechanical hyperalgesia and that calcitonin gene-related peptide (CGRP) expression in the deeper lamina layer of

59 the spinal dorsal horn increases in rats that have been immobilized for 8 weeks. These
60 findings suggest that it is necessary to keep the affected limb as active as possible
61 during the acute phase of inflammation.
62 Previously, it has been demonstrated that exercise has an analgesic effect and prevents
63 development of chronic pain (Stagg *et al.* 2011, Detloff *et al.* 2014). In an experimental
64 animal model, the use of intensive training using forced exercise wheel walking system
65 after spinal cord injury can prevent the development of neuropathic pain (Detloff *et al.*
66 2014). Aerobic exercise training has been shown to reduce hyperalgesia following
67 injury (Stagg *et al.* 2011). However, the biological effects of passive joint motion on
68 pain have not yet been reported. Continuous passive motion (CPM) is a treatment
69 protocol that is used to manage the ROM after joint surgery and/or treatment of the
70 inflamed joint. CPM has biological effect on the healing and regeneration of articular
71 tissues with better histologic properties compared to immobilization (Salter *et al.*
72 1981). Additionally, the expression of IL-1β and COX-2 decreases with CPM in rat
73 meniscal chondrocytes immediately following inflammation whereas the expression of
74 IL-10 increases (Ferretti *et al.* 2005). These results explained the molecular basis of
75 the beneficial effect of CPM when applied during the acute phase of joint inflammation.
76 Therefore, we hypothesized that CPM has a positive effect on the reduction of acute
77 pain originating from inflammation and alleviating the development of secondary
78 hyperalgesia. This study aimed to examine the effect of CPM that was initiated after

the onset of arthritis on inflammation and secondary hyperalgesia in a rat model.

Methods

Animals

The Ethics Review Committee for Animal Experimentation at the authors' current institution approved all experiments. Male Wistar rats (8 weeks old, Kyudo, Saga, Japan) were used for the experiments.

All rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), and subsequently given a single 300-μL injection of a mixture of 3% kaolin and 3% carrageenan (Sigma Chemical Co, St. Louis, USA) into the right knee joint anteriorly, and then randomly divided into the following 3 groups: (1) control (n = 8), (2) immobilization (IM; n = 9), and (3) immobilization with CPM (CPM; n = 8). The rats in the control group were untreated. All rats were housed in a 12-h light/dark cycle. Behavioral testing was usually done between 9 AM and 5 PM. Food and water were available ad libitum. All treatments mentioned below were administered under pentobarbital sodium anesthesia (40 mg/kg).

Immobilization

The right knee joints of rat in the IM and CPM group were immobilized in full extension, the ankle joints were immobilized in full planter flexion using a plaster cast, whereas the left leg remained free. The plaster cast was wrapped from the pelvic area to the right distal foot while their right hind limbs were kept ramrod-straight. The

plaster cast was replaced at least every 2 or 3 days to prevent loosening and edema in the hind paw. The rats were able to move freely in the cage by using the three limbs that were not immobilized.

Range of Motion on Knee Joint Flexion

ROM was measured with a goniometer. It was defined as the angle of a straight line connecting the great trochanter and the center of the knee joint to a line connecting the center of the knee joint and the malleolus lateralis of the fibula when the knee was flexed passively under a tensile force of 0.3 N using a spring scale, as described previously (Honda *et al.* 2015).

Continuous Passive Motion

In previous studies, CPM for 60 minutes once a day provided better pain response in patients with a frozen shoulder (Dundar *et al.* 2009). CPM treatment for 6 days per week increased the active ROM of patient after total knee arthroplasty (Lau and Chiu 2001). In this study, CPM was applied for 60 min, once a day, 6 days a week for 56 days from the day after injection. The knee joints were flexed to an angle equivalent to the angle recorded at maximum flexion, at an angular velocity of 10 degrees/s using a mechanical ankle stretcher (Sakai Iryo, Osaka, Japan). ROM was adjusted in each individual rat using the above-mentioned method. Following completion of daily treatment, right knee joints were re-immobilized with a plaster cast.

Knee Joint Swelling

In order to follow the changes in joint swelling over time, we measured the transverse diameter of the right knee joint using a manual caliper. Rats were placed individually in a homemade restrainer (Nakano *et al.* 2012) so that loading on the right hind limb was avoided and the knee joint was held in the maximum extended position.

Pressure Pain Threshold in the Knee Joint

The pressure pain threshold (PPT) of the inflamed knee joint was assessed using a Randall-Selitto apparatus (Ugo Basile, Varese, Italy). Rats were lightly restrained by hand. The rounded tip of the transducer probe (base diameter = 9 mm) was applied to the lateral side of the knee joint with linearly increasing pressure (48 g/s). The threshold was defined as the force required for eliciting the hind limb flexion reflex or vocalization. Five measurements were taken at intervals of at least 5 minutes, and the average of three measurements (excluding the maximum and minimum) was recorded as the PPT.

Paw Withdrawal Response

Mechanical hyperalgesia of the hind paw was tested with von Frey filaments. The animals were placed individually in a homemade restrainer mentioned above. This technique was employed because ROM limitations of the hip, knee, and ankle joint prevented the immobilized rats from placing their right hind paws on the ground. After removing the plaster cast, the rats were allowed to acclimate for 20 min before testing. The glabrous skin of the hind paw was probed 10 times using 4 g and 15 g von Frey

filaments (VFFs; North Coast Medical, Morgan Hill, CA, USA) every 10 s. Lifting or pulling back of the paw or vocalization was counted as the paw withdrawal response (PWR) by a single experimenter. The 4 g and 15 g filaments were used to ascertain mechanical allodynia and hyperalgesia, respectively (Peleshok and Riberio-da-Silva, 2011).

Analysis of Calcitonin Gene-Related Peptide in the Spinal Dorsal Horn

In this study, we focused on the expression of CGRP in the spinal dorsal horn, which may play a role in central sensitization (Kangrga and Randic 1990).

At the end of the immobilization period, all the rats were anesthetized. The spinal cord (L2-3, L4-5) was removed following the transcardial perfusion of saline and 4% paraformaldehyde dissolved in a 0.1 M phosphate-buffer (PB; pH. 7.4). The tissue was soaked for 24 h in 10% sucrose dissolved in PB, followed by 24 h in 20% sucrose dissolved in 0.01 M phosphate-buffered saline (PBS; ph7.4). Spinal cord frozen sections (10 μm) were cut with a cryostat. In order to inhibit endogenous peroxidases, the sections were incubated for 30 min at room temperature with 0.3% H_2O_2 dissolved in methanol. Next, sections were blocked for 20 min with 5% bovine serum albumin dissolved in PBS, followed by incubation with an anti-CGRP polyclonal antibody (1:500 rabbit; ImmunoStar Inc. Hudson, WI, USA) overnight at 4 °C. Subsequently, they were incubated with goat anti-rabbit IgG conjugated to Texas Red® (1:600, Vector labs, CA, USA) for 1 h at room temperature. Quantitative evaluation of the calcitonin

159 gene-related peptide (CGRP) expression in the ipsilateral dorsal horn was performed
160 using image-analysis software (NIS-Element Ver.3, Nikon Instruments Inc., NY, USA).
161 The spinal dorsal horn was divided into the superficial (lamina I-II) and deeper layers
162 (lamina III-VI), according to previously described criteria (Molander *et al.* 1984). The
163 intensity of CGRP expression reflected the quantity of fluorescence observed in the
164 superficial (lamina I-II) and deeper (lamina III-VI) layers of the spinal dorsal horn in 5
165 sections per tissue.

166 Statistical Analysis

167 All data are presented as mean ± SE. Differences between groups were assessed using
168 the one-way analysis of variance followed by Fisher's protected least significant
169 difference post hoc test. Differences were considered significant at $p < 0.05$.

170 Results

171 Changes in Knee Joint Swelling

172 The ipsilateral knee joint transverse diameter increased significantly 1 day after
173 injection in all groups compared to baseline. There were no significant differences in
174 the diameter among the groups at day 1 (Fig. 1). In the IM group, there were no
175 significant differences at each testing point compared to the control group. Similarly,
176 there was no significant difference between the CPM and control groups except at 56
177 days after injection.

178 Pressure Pain Threshold

The PPT 1 day after injection was significantly lower in all groups compared to baseline (Fig. 2). In the control group, the PPT was recovered 14 days after injection. The PPT in the IM group was significantly decreased compared to the control group at 14 days after injection and then remained steady until 56 days after injection. In contrast, there were no significant differences between the control and CPM groups until 21 days after injection, at which point a significant increase was found compared with the IM group from 14 to 35 days after injection. However, the PPT in the CPM group significantly decreased compared with the control group on or after day 28. Moreover, there were no significant differences between the IM and CPM groups on or after day 42.

Paw Withdrawal Threshold

The PWR of all rats, as measured by 4 g von Frey filaments for mechanical allodynia, significantly increased on the 1 day after injection compared to the baseline (Fig. 3A). In the IM group, a significant increase in the PWR was found from 21 to 56 days after injection compared to the control group. In contrast, the PWR in the CPM group was not significantly different from that of the control group, except at 21 and 42 days after injection. As measured by 15 g von Frey filaments for mechanical hyperalgesia, a remarkable increase in the PWR was identified in the IM group at 14 days after injection, which persisted for 42 days (Fig. 3B). The PWR of the CPM group was significantly decreased 14 to 56 days after injection, and no significant difference was

199 confirmed for 35 days following injection, when compared to the control group.

200 Expression of CGRP in the Spinal Dorsal Horn

201 The CGRP immune response in the deep layer of the ipsilateral dorsal horn (L2-3) was

202 greater in the IM and CPM groups compared to the control group (Fig. 4A). CGRP

203 expression intensity analysis revealed no significant differences among the three

204 groups in the superficial layer (laminae I - II) (Fig. 4C). Although CGRP expression

205 intensity was significantly higher in the IM and CPM groups compared to the control

206 group in the deep layer (laminae III - VI) (Fig. 4D), there were no significant

207 differences between these two groups. In the superficial layer of the ipsilateral dorsal

208 horn (L4-5), the CGRP expression intensity of the IM group was significantly higher

209 than that of the control group (Fig. 4B). However, there were no significant differences

210 between the control and CPM groups (Fig. 4E). Although the intensity of the IM and

211 the CPM groups was significantly higher than that of the control group in the deep

212 layer of the dorsal horn, no significant differences were noted between the IM and the

213 CPM groups (Fig. 4F).

214 ROM on Knee Joint Flexion

215 ROM of right knee flexion was decreased in all groups on the first day after injection.

216 In the control group, this change was transient (Fig. 5). ROM in the IM and CPM

217 groups continued to decrease until 56 days after injection. However, ROM in the CPM

218 group significantly increased compared to the IM group from 3 to 56 days after

injection.

Discussion

The current study examined the effects of CPM that was initiated after the onset of arthritis on inflammation and pain-related behavior in rats. In previous studies, the carrageenan model was commonly used for experimental joint inflammation (Okamoto *et al.* 1999, Radhakrishnan *et al.* 2003) because of plasma extravasation after the release of neuropeptides (Lam and Ferrell 1993) and other inflammatory mediators, such as prostaglandins (Nantel *et al.* 1999), and bradykinin (Birrell *et al.* 1993)). These noxious chemicals sensitize primary afferent fibers resulting in primary and secondary hyperalgesia (Schaible *et al.* 1988, Radhakrishnan *et al.* 2003). Therefore, this arthritis model is favorable for the examination of the effects of CPM on inflammation and secondary hyperalgesia.

In the present study, the results of knee joint transverse diameter and PPT at 1 day after injection indicated that injection of carrageenan produced acute inflammation and primary hyperalgesia in the affected joint. Moreover, these results indicated that injection produced the same level of arthritis in all animals.

The PPT in the control group increased to a level higher than the baseline after day 21. Previous study reported that the increase of the PPT in rats was associated with the alteration of their body weight gain (Luis-Delgado *et al.* 2006). In this study, the rats of the control group gained their weight gradually with aging (date are not shown).

239 Therefore, the PPT in the control group increased with the growth of the rat. The PPT
240 in the IM group was significantly decreased compared to the control group at 14 days
241 after injection, which indicates that immobilization after induction of inflammation
242 prolongs the recovery of mechanical hyperalgesia. In contrast, a significant increase in
243 the PPT was confirmed in the CPM group compared to the IM group. Additionally,
244 there were no significant differences between the CPM and control groups. Our
245 findings revealed that CPM initiated after the onset of arthritis promotes the recovery
246 of inflammatory-induced primary hyperalgesia. The CPM performed immediately after
247 the induction of knee joint inflammation inhibited the expression of inflammatory
248 cytokine and induced the expression of the anti-inflammatory cytokine (Ferretti *et al.*
249 2005). In this study, CPM of inflamed joints may have evoked a beneficial biological
250 reaction that promoted the recovery of primary mechanical hyperalgesia. A significant
251 decrease in the PPT was sustained in the IM group until day 56. Beginning 28 days
252 after injection, the PPT in the CPM group was significantly decreased compared to the
253 control group. In addition, there were no significant differences in the PPT between
254 the IM and CPM groups from 42 to 56 days after injection. The knee joint
255 immobilization for 6 week enhanced the medial articular nerve activity in rabbits
256 during rest and knee joint motion to levels similar to those found in inflamed knee
257 joints (Okamoto *et al.* 1999). Immobilization of the rat forelimb for 4 week produced
258 mechanical allodynia that was related to plastic changes in the dorsal horn neuron

(Ushida and Willis 2001). Our results showed that long-lasting immobilization negated the beneficial effects of CPM and produced hyperalgesia derived from plastic changes in the neurons.

Although the CGRP expression intensity in the deep layer (laminae III - VI) of L2-3 was significantly higher in the IM and CPM groups compared to the control group, there were no significant differences between these two treatment groups. It is known that CGRP increases the discharge frequency of WDR neurons in the dorsal horn, which is blocked by the CGRP receptor antagonist CGRP8-37 (Yan *et al.* 2004). This indicates that increased CGRP expression in the spinal dorsal horn reduces the pain threshold through activation of WDR neurons, which are distributed in the deep layer of the spinal dorsal horn. Therefore, we considered that the decrease in PPT in the IM and CPM groups, at least after 56 days, was induced by central sensitization.

In this study, sensitivity of the hind paw was tested with 4 g von Frey filaments for mechanical allodynia and 15 g filaments for mechanical hyperalgesia. Allodynia is defined as a pain due to a stimulus that does not normally provoke pain, and hyperalgesia is an increased response to a stimulus which is normally painful. The increased PWR at 1 day after injection in all groups demonstrated mechanical allodynia and hyperalgesia in locations distal from an inflamed joint. Injection of carrageenan into deep tissues activates the dorsal horn neurons causing central sensitization (Neugebauer and Schaible 1990). Central sensitization is usually

observed in the areas adjacent to the injury and sometimes in distal locations (Radhakrishnan *et al.* 2003), manifested as secondary hyperalgesia (Sluka and Westlund 1993). In the IM group, mechanical allodynia and hyperalgesia in the hind paw were sustained until 56 days after injection. In contrast, in the CPM group, mechanical allodynia in the hind paw was not seen from 49 to 56 days. Moreover, mechanical hyperalgesia was not seen until 35 days after injection. This may indicate that CPM reduces mechanical allodynia and hyperalgesia in arthritis and may inhibit central sensitization in the spinal dorsal horn. However, after 42 days injection, secondary hyperalgesia in the CPM group were confirmed, which was mild compared to that of the IM group. Previous study demonstrated that eight-week joint immobilization induced hyperalgesia to mechanical stimulation associated with central sensitization in the spinal cord (Hamaue *et al.* 2013). This may influence the decrease in the pain threshold in the CPM group. Therefore, secondary hyperalgesia in the IM and CPM groups is caused by inflammation or immobilization.

In the superficial layer (laminae I-II) of the spinal dorsal horn in L4-5, CGRP expression intensity was significantly higher in the IM group compared to the control group, whereas no significant differences were seen between the control and CPM groups. Although CGRP expression intensity in the deep layer (laminae III-VI) of the spinal dorsal horn was significantly increased in the IM and CPM groups compared to the control group, there were no significant differences between the treatment groups.

299 CGRP released into the superficial layer (laminae I-II) of the spinal dorsal horn
300 induces hypersensitivity via the increased release of substance P and other
301 neuropeptides (Kangrga and Randic 1990, Sun *et al.* 2004). Our results indicate that
302 CPM inhibits the central sensitization induced by immobilization during the acute
303 phase of arthritis. This is the one of the reasons why mechanical hyperalgesia in the
304 CPM group was mild compared to the IM group. It was found that the mobilization of
305 the inflamed knee joints of rats at 4 weeks increased the mechanical withdrawal
306 threshold (Sluka *et al.* 2006). The authors discussed the possibility of the involvement
307 of non-opioid pathways in the descending inhibition using serotonin and noradrenaline
308 to produce analgesia. In the current study, descending inhibition may affect the
309 decrease in secondary hyperalgesia.
310 One day after injection, the ROM was significantly decreased compared to those at
311 baseline in all groups, with significantly increased ipsilateral knee joint transverse
312 diameter, which indicated knee joint swelling by acute inflammation. Increases in the
313 synovial fluid induced an increase in the intra-articular pressure and a decrease in the
314 joint angle (Wood *et al.* 1988). Therefore, the limitations in ROM on 1 day after
315 injection can be attributed to the development of swelling of the knee joint. The ROM
316 in the IM and CPM groups was significantly decreased from 3 to 56 days after injection
317 compared to the control group. The time-dependent limitation in ROM in the IM and
318 CPM groups was induced by cast immobilization, which is derived from myogenic

(Honda *et al.* 2015) and arthrogenic changes (Akeson *et al.* 1973). The ROM in the CPM group was significantly increased compared to the IM group. These results suggest that CPM is useful for the management, not only of hyperalgesia, but also of ROM limitations.

In summary, CPM initiated after the onset of arthritis promotes the recovery of inflammatory primary hyperalgesia and prevents the development of secondary hyperalgesia by decreasing CGRP expression in the superficial layer of the spinal dorsal horn. Additionally, CPM can inhibit progression of the immobilization-induced joint contracture. Therefore, we believe that CPM initiated after the onset of arthritis is beneficial for acute and chronic pain management. However, this study has some limitations. First, this research evaluated the knee joint transverse diameter and pressure pain threshold according to the severity of arthritis. In order to clarify the effects of CPM on inflammation, alteration of inflammatory cytokines and histological changes in the synovium must be quantified from the acute phase. Second, we only examined the expression of CGRP in the spinal dorsal horn. However, there are many factors involved in the central sensitization such as activation of the glial cell and expression of other neurotransmitters, substance P, nitric oxide, and glutamate. Additionally, the analysis of CGRP was performed with only experimental endpoints. In order to elucidate the mechanism of central sensitization and biological mechanism of CPM for decreased primary and secondary hyperalgesia, further research is

339 necessary.

340 Conflict of Interest

341 There is no conflict of interest.

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430 neurons in the spinal dorsal horn of rats. *J Neurosci Res* **77**: 148-152, 2004.

431 Figure captions and legends

432 Fig. 1. Time course changes in the transverse diameters of the knee joints. Data are
433 presented as the mean ± SE. *p < 0.05 continuous passive motion (CPM) versus the
434 control (CON) group. †p < 0.05 immobilization (IM) versus CPM group. B, baseline.
435 Fig. 2. Time course changes in the pressure pain thresholds of the knee joints. Data are
436 presented as the mean ± SE. *p < 0.05 continuous passive motion (CPM) or
437 immobilization (IM) versus the control (CON) group. †p < 0.05 IM versus CPM group. B,
438 baseline.

439 Fig. 3. Time course changes in the paw withdrawal thresholds of the ipsilateral hind
440 paws. (A) 4 g von Frey filament (VFF) as a measurement of mechanical allodynia. (B)
441 15 g VFF as a measurement of mechanical hyperalgesia. Data are presented as the
442 mean ± SE. *$p < 0.05$ continuous passive motion (CPM) versus the control (CON) group.
443 †$p < 0.05$ immobilization (IM) versus CPM group. B, baseline.
444 Fig. 4. Intensity of calcitonin gene-related peptide (CGRP) expression in the ipsilateral
445 dorsal horn of the spinal cord. Representative photomicrographs of CGRP
446 immunohistochemistry in the ipsilateral dorsal horn are shown, at the L2-3 (A) and
447 L4-5 levels (B). The CGRP-positive neural fibers were clearly observed in the deep
448 layer of the dorsal horn in the immobilization (IM) and continuous passive motion
449 (CPM) groups (arrowheads). Percentage control of fluorescence intensity of CGRP
450 expression in the superficial (laminae I-II) (C, E) and deep layers (laminae III-VI) were
451 calculated (D, F) in the L2-3 (C, D) and L4-5 (E, F). Data are presented as the mean ±
452 SE. *$p < 0.05$ versus the control (CON) group. Scale bars = 100μm
453 Fig. 5. Time course changes in the range of motion (ROM) of the knee joints on flexion.
454 Data are presented as the mean ± SE. *$p < 0.05$ continuous passive motion (CPM)
455 versus the control (CON) group. †$p < 0.05$ immobilization (IM) versus CPM group. B,
456 baseline.
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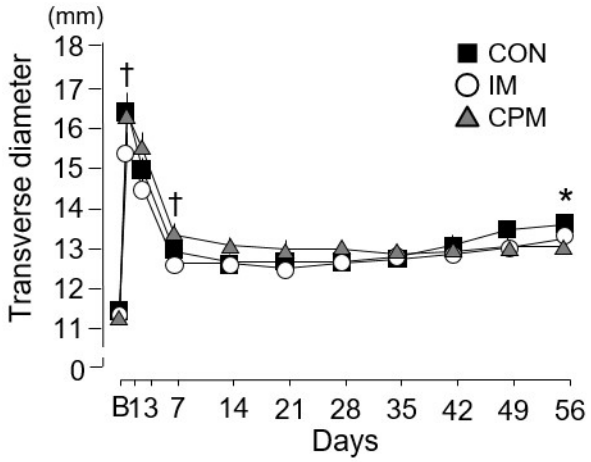
458

459 Fig.1



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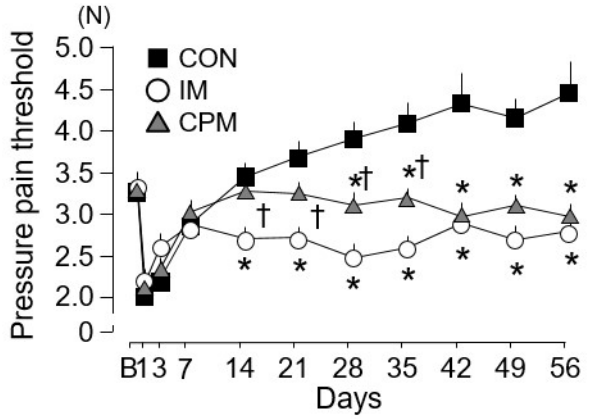
462 Fig.2



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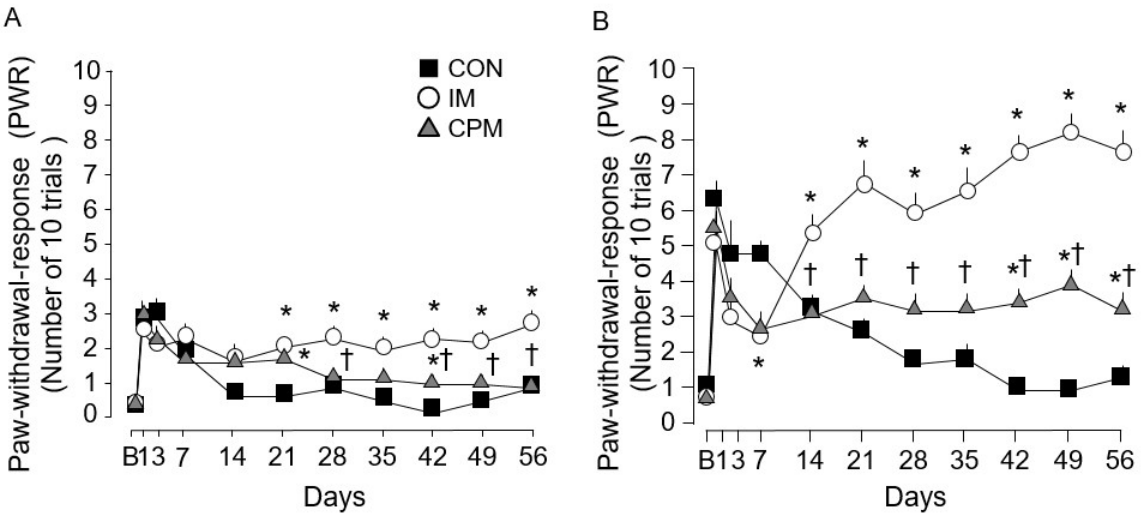
470 Fig.3



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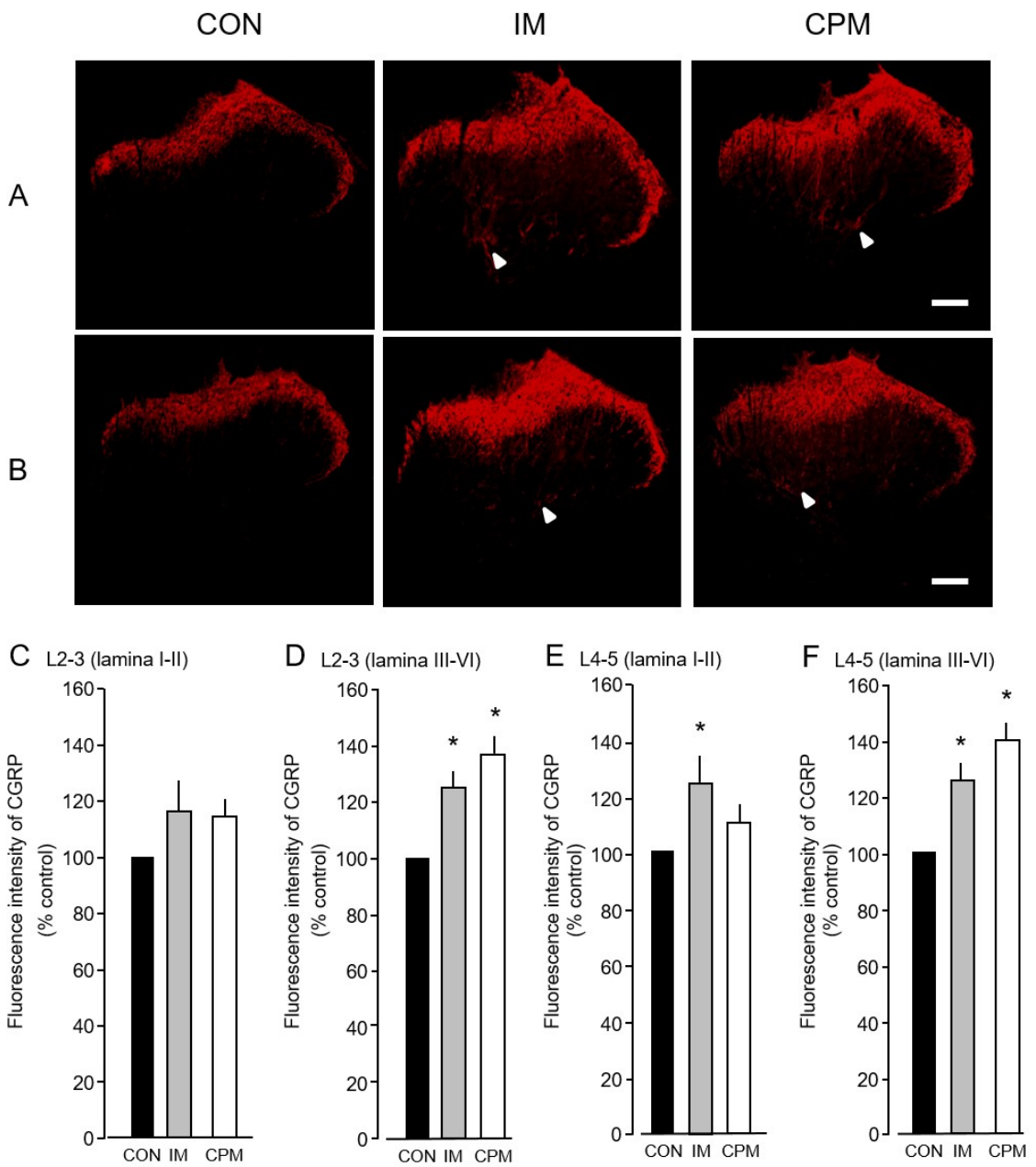
484 Fig.4



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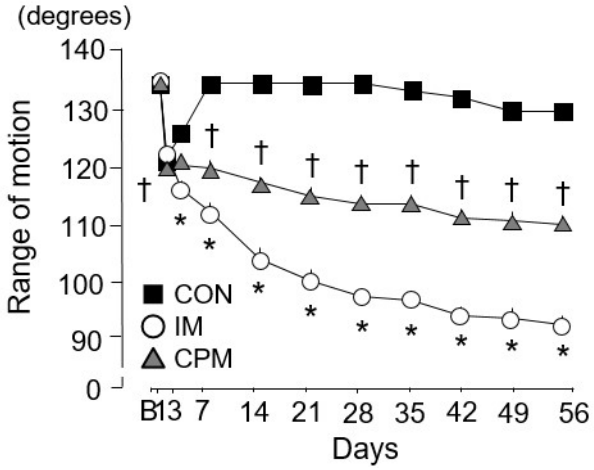
490 Fig.5


(degrees)
140
130
120
110
100
90
0
Range of motion
CON
IM
CPM
B
1
3
7
14
21
28
35
42
49
56
Days

491