weaker filaments were applied one by one until the animal did not show withdrawal response. Then a stronger filament was applied again. This up and down procedure was repeated a few times to decide the withdrawal threshold. A filament was applied twice and if an animal showed at least one withdrawal response, this was taken as positive. Each filament was applied at a few second intervals.

As repeated acid injections into gastrocnemius muscle caused bilateral secondary hyperalgesia of the plantar surface of the paw (Sluka *et al.* 2001), we also examined von Frey hair threshold of the plantar surface of the paw bilaterally.

Other procedures were the same as the Randall-Selitto test.

After completion of a series of behavioral pain tests, the animals used were euthanased by inhalation of carbon dioxide.

C-Fos study

Exercise and compression protocol (Fig. 1*B*)

On day 2 the animals in the ECC and SHAM groups were divided into 2 respective subgroups, with or without muscle compression. Thus, there was a total of four groups (n=17): a sham exercised group without muscle compression on day 2 (SHAM, n=4), a sham exercised group with muscle compression (SHAM+compression, n=4), an eccentrically exercised group without muscle compression (ECC, n=5), and an eccentrically exercised group with muscle compression (ECC+compression, n=4). Compressive stimulation was applied to the exercised muscle through the skin on day 2. Compression with a force of 1568 mN was applied by the Randall-Selitto apparatus

(Ugo Basile, Italy) for 10 sec, followed by a 20 sec rest period. This was repeated for 30 minutes under anesthesia (sodium pentobarbital, 50 mg/kg, i.p.). The force of 1568 mN is about 2 times higher than the withdrawal threshold in awake rats (described below).

Effect of Morphine

In another series of experiments, the effect of morphine on c-Fos expression induced by ECC+compression was examined to make sure that c-Fos expression was induced by noxious inputs (Presley, et al., 1990). Eleven animals were used in this series. The methods and interval for ECC and compression were the same as described above. Five animals received morphine (10 mg/kg, dissolved in saline in 10 mg/ml, i.p.) 20 min before compression of EDL, and the remaining 6 received saline (1 ml/kg, i.p.) instead of morphine.

Immunohistochemistry

Two hours after the end of the muscle compression (or 2 days after the exercise session in groups without compression), the animals were deeply anesthetized with sodium pentobarbital, and then perfused transcardially with 500 ml of 0.1 M phosphate buffered saline (PBS, pH 7.4) followed by 500 ml of 4 % paraformaldehyde in 0.1 M PBS. The spinal cord (L1-S1) was quickly removed, post-fixed for 24 h in the same fixative, and then cryoprotected in 15% sucrose dissolved in PBS for 1 day, followed by 30% sucrose for 2 days. Transverse frozen sections (40 μm) of the spinal cord from L2 to L6 were cut with a microtome, and every second section was used for analysis.

Immunohistochemical staining of c-Fos was performed according to the avidin-biotin

peroxidase method (Hsu *et al.* 1981). Briefly, following several rinses in PBS, endogenous tissue peroxidase activity was quenched by soaking the sections in 0.3% hydrogen peroxide solution in PBS for 30 min. The specimens were then treated in blocking solution, PBS containing 0.3% Triton X-100, and 5% normal goat serum (DGS00301, Cosmo Bio Co., Ltd. Japan) for 2 h. They were processed for immunohistochemistry by free-floating ABC technique using polyclonal rabbit antibody to c-Fos (1:5000, sc-52, Santa Cruz Biotechnology, Santa Cruz, USA) diluted with the blocking solution described above for 3 days at 4°C, biotinylated goat anti-rabbit immunoglobulin (1:500, BA-1000, Vector Laboratories, USA) for 90 min, and avidin-biotin complex (PK-6100, Vector Laboratories, USA) for 90 min at room temperature. Sections were washed with PBS between incubations. Finally, sections were reacted in a 0.05 M Tris-buffer (pH 7.6) containing 0.02% 3,3'-diamino-benzidine tetrahydrochloride (DAB), 0.02% nickel ammonium sulfate and 0.003% hydrogen peroxide for a few minutes at room temperature to produce a purple-black reaction product. The sections were washed in the Tris-buffer to stop the staining reaction, and then mounted on gelatin-coated slides, air-dried, cleaned in xylene and cover-slipped with mounting medium (Mount Quick, Daido Sangyo Co., Ltd, Japan).

For quantitative analysis, the number of c-Fos-ir neurons was counted under a light microscope at a magnifying power of 200. To study the laminar distribution of c-Fos, the spinal dorsal horn was divided into 3 specific regions based on the cytoarchitectonic organization reported by Molander *et al.* (1984): the superficial dorsal horn (SDH, lamina I-II), the proprius nucleus (PN, lamina III-IV), and the neck of the dorsal horn (NDH, lamina V-VI). The investigator responsible for counting the c-Fos-ir neurons was

blinded to the group to which a section belonged. The number of c-Fos-ir neurons in an animal was divided by the number of sections counted and the result represented the average number of cells per section for a certain animal.

Statistical analyses

Results are expressed as mean ± SE. One-way ANOVA followed by Bonferroni's multiple comparison test was performed to examine the change in the withdrawal threshold measured by the Randall-Selitto test. Withdrawal threshold measured by the Randall-Selitto test was compared on each day between SHAM and ECC groups with an unpaired t test. Non-parametric Friedman test followed by Dunn's post-hoc test was used to compare the withdrawal threshold measured by von Frey hair test, and the non-parametric Mann-Whitney test was used to compare the values after ECC between SHAM and ECC groups. We used these different statistical methods for the values obtained with the Randall-Selitto test and von Frey hair test, because the former values were continuous, whereas the latter were discontinuous and increased in logarithmic order. In the c-Fos study one-way ANOVA followed by Bonferroni's multiple comparison test was used to compare all pairs among the 4 groups. $P<0.05$ was considered to be significant.

Results

Mechanical hyperalgesia

The mechanical threshold measured with the Randall-Selitto apparatus was almost stable before exercise, at 912.1 ± 27.3 mN for the sham exercised animals and 946.4 ± 36.7 mN for ECC animals 1 day before exercise. This value started to decrease in ECC

animals 1 day after exercise, and reached a minimum 2 days after exercise (Fig. 2*A*). Despite this change in withdrawal threshold, no apparent changes in spontaneous behaviours were recognized. Decreased mechanical withdrawal threshold, although less intense, was still observed 3 days after exercise. Complete recovery was observed 7 days after exercise. The change in the withdrawal threshold in ECC was statistically significant 1, 2, and 3 days after exercise compared with that 1 day before exercise (day -1 in Fig. 2*A*, $p<0.05\sim0.001$). The withdrawal threshold of the sham-exercised animals remained constant during the entire experimental period (Fig. 2*A*), and the withdrawal threshold of ECC animals was significantly lower than the sham-exercised group 1, 2 and 3 days after exercise ($p<0.01\sim0.001$).

Despite of the decrease in mechanical withdrawal threshold, no pain related behaviours, such as licking, biting, lifting of the exercised paw, were observed. And they walked normally in their cage up to 7 days after eccentric exercise.

In contrast, the pain threshold at the skin over the EDL muscle, which was measured as the withdrawal threshold to VFH stimulation, showed no significant change after treatment (eccentric or sham muscle contraction) in either the ECC or the SHAM group ($p>0.05$ when the thresholds after treatment were compared with that on day -1, Friedman test followed by Dunn's multiple comparison test; $p>0.05$ when the threshold was compared between 2 groups on each day after treatment, non-parametric Mann-Whitney test, Fig. 2*B*), suggesting that the change of the withdrawal threshold measured with the Randall-Selitto apparatus represents the pain threshold change in deeper tissues, possibly the muscle.

Bilateral von Frey hair threshold of the plantar surface of the paw did not change significantly for at least 7 days after eccentric exercise (Von Frey hair thresholds of the ipsilateral plantar surface of the skin were 237.9±25.1 mN, 249.8±15.7 mN and 244.0±25.6 mN immediately before (-1), 2 and 7 days after eccentric exercise, respectively, and those of the contralateral plantar surface of the skin were 244.0±25.6 mN, 256.2±24.1 mN and 207.6±24.2 mN, respectively, n=7).

C-Fos study

Representative sections of each group are shown in Fig. 3. C-Fos-ir was found in nuclei of some neurons in the dorsal horn of the spinal cord (sample photograph of the ECC+compression group in Fig. 3*F*). The number of c-Fos-ir neurons in the dorsal horn was small in the SHAM group (Fig. 3*A*). Similarly, only a few neurons were labelled in the ECC and SHAM+compression groups (Fig. 3*B, C*). Thus, neither ECC 2 days before by itself nor the compression (two times the Randall-Selitto threshold) used in this experiment activated the nociceptive pathway significantly in anesthetized animals. In the ECC+compression group, on the other hand, c-Fos immunoreactivity clearly increased, especially in the middle of the superficial dorsal horn corresponding to lamina I/II (Fig. 3*D, E, F*). A smaller number of c-Fos-ir neurons was seen also in the proprius nucleus in the case shown in Fig. 3. Figure 4 shows the segmental distribution of c-Fos-ir positive neurons in one rat of the ECC+compression group. The most prominent increase in c-Fos expression was observed in the spinal cord at L4, and some expression was also found in L2-L3 spinal segments as seen in this case, but not L5 and L6 (Fig. 4*A*). On average in four cases, the number of c-Fos-ir positive neurons in the

entire dorsal horn (lamina I-VI) at the L4 segment on the ipsilateral side was significantly greater in the ECC+compression group than in the SHAM group ($p<0.01$, one-way ANOVA followed by Bonferroni's multiple comparison test, Fig. 4*B*). It must be noted that the number of c-Fos-ir positive neurons in the ECC+compression group was also greater than in the other two groups ($p<0.01$, one-way ANOVA followed by Bonferroni's multiple comparison test, Fig. 4*B*). ECC and SHAM+compression groups had numbers of c-Fos-ir positive neurons that were not significantly different from the SHAM group. At L2 and L3, the number of c-Fos-ir neurons tended to be greater in the ECC+compression group than in the other three groups, but none of the differences among the 4 groups were significant. The number of c-Fos-ir neurons occasionally looked increased in the contralateral side in the ECC+compression group, especially in cases with larger numbers of c-Fos-ir neurons (example in Fig. 4*A*), but the change was not statistically significant compared with the other groups (Fig. 4*B*).

Closer inspection of the spinal dorsal horn at L4 (Fig. 5*A*) reveals a clear increase in the numbers of labelled neurons in the superficial dorsal horn (SDH, lamina I-II) in the ECC+compression group (ipsilateral SDH Fig. 5*A*, 1.9 ± 0.6 neurons/section (n=4) in the SHAM group vs. 15.6 ± 3.7 neurons/section (n=4) in the ECC+compression group, $p<0.01$). The number of c-Fos-ir neurons in the ECC+compression group was also significantly greater than in the other two groups ($p<0.01$ and 0.001). The majority of c-Fos-ir neurons were found in the middle or medial half of the superficial dorsal horn (Fig. 3*D, E, F* & Fig. 4*A*) corresponding to the projection area of input from the common peroneal nerve (Brushart *et al.* 1981; Molander & Grant. 1986). The numbers of labelled neurons in the ECC and SHAM+compression groups were not significantly

different from that in the SHAM group.

There was a tendency that the number of c-Fos-ir neurons in the proprius nucleus was larger in ECC+compression group (PN in Fig. 5*A*, 4.5 ± 1.4 neurons/section (n=4) in the ECC+compression group vs. 1.6 ± 1.0 neurons/section (n=4) in the SHAM group), but the difference was not significant.

Effect of morphine

In this series of experiments, ECC+compression induced expression of c-Fos immunoreactivity in almost the same number of dorsal horn neurons (saline group, Fig. 5*B*) on the side ipsilateral to the stimulation as in the experiment described above. These neurons were found mainly in the superficial dorsal horn. Morphine treatment clearly decreased the number of c-Fos-ir neurons in the dorsal horn (morphine group in Fig. 5*B*, $p<0.01$ compared with saline group).

Discussion

In the present experiment the mechanical pain threshold after ECC decreased when measured with the Randall-Selitto apparatus, but not when measured with VFHs. Because the tip diameter of the VFHs is small (0.5 mm), it is unlikely that the mechanical stress applied to the surface is transmitted deep into the muscle (Takahashi *et al.* 2004); thus the VFH measures the pain threshold of the skin. In contrast, the tip of the Randall-Selitto apparatus is much larger than that of von Frey hairs, and so the force applied can be transmitted deeper through the skin (Takahashi *et al.* 2004). Therefore, together with the absence of change in the VFH pain threshold, change in the pain

threshold measured with the Randall-Selitto method is considered to represent the change in the pain threshold in the deeper tissue, probably the muscle. This change was observed 1 day after ECC and reached the lowest value 2 days after ECC, which is compatible with our everyday experience of delayed onset muscular soreness (Armstrong, 1984; Newham, 1988; Proske & Morgan, 2001).

The present results clearly demonstrated that c-Fos expression was increased only in the ECC+compression group. In the ECC group, there were a few labelled neurons in the dorsal horn at L4, but the number did not differ from the SHAM group. This observation is consistent with the fact that spontaneous pain is not prominent in DOMS 2 days after eccentric contraction (Graven-Nielsen & Arendt-Nielsen, 2003). Because c-Fos expression is known to be induced by many factors in corresponding neural pathways, one might expect that muscular contraction or stretching stimulation itself induces c-Fos. However the expression levels of c-Fos protein in neurons usually peak about 2 h after neuronal excitation and expression disappears 4-16 h following excitation (Menétrey *et al.* 1989). In the present study the animals underwent either muscle stretching or muscle contraction by electrical stimulation of the nerve 2 days before perfusion. Therefore, any c-Fos-ir expression that had been induced by these stimuli should have already vanished by the time the animals were perfused. In the SHAM+compression group, the number of labelled neurons did not differ from the SHAM group. This result suggests that muscle compression itself did not activate the pain pathway in the anesthetized condition.

In contrast, the number of neurons with c-Fos-ir in the ECC+compression group

significantly increased, and the increase was observed in the ipsilateral superficial dorsal horn at L4, when compared with the other 3 groups. The superficial dorsal horn is well known to be an important region where thinly myelinated and unmyelinated afferents from skin (Light & Perl, 1979; Sugiura *et al.* 1986), viscera (Cervero & Connell, 1984; Mizumura *et al.* 1993) and muscle (Brushart & Mesulam, 1980; Brushart *et al.* 1981; Mense & Craig, 1988; Ling *et al.* 2003) terminate. Brushart *et al.* found that muscle afferents of the rat common peroneal branch projected densely to the substantia gelatinosa (Brushart *et al.* 1981). Recently Ling et al. demonstrated, by intracellular injection of *Phaseolus vulgaris leucoagglutinin* to single C-afferent neurons in dorsal root ganglia, that unmyelinated (C) afferent fibres from the medial head of the gastrocnemius muscle run at the surface of the dorsal funiculus, giving off collaterals into laminae I and II, and sometimes into parts of lamina III (Ling *et al.* 2003). Thus, the prominent increase of c-Fos-ir in the superficial dorsal horn of the ECC+compression group and absence of increase in SHAM+compression group strongly suggest the muscle was hyperalgesic to mechanical stimulation (existence of tenderness) 2 days after eccentric muscular work. This conclusion was supported by the fact that morphine treatment clearly decreased c-Fos immunoreactivity in the dorsal horn.

The increase of c-Fos-ir positive neurons in the ECC+compression group was found mainly in L4, and some in L3 to L2, spinal segments; no increase was found in L5 or further caudally. A previous report showed that the great majority of sensory neurons innervating EDL are located in L4 dorsal root ganglion (Peyronnard *et al.* 1986). Spinal terminations of the gastrocnemius muscle traced by intracellular labelling technique

were found rostral to the level where fibres entered into the spinal cord, but not caudal to it (personal communication from Dr. Y. Sugiura). The present result is in agreement with this observation, and may suggest that muscle afferents terminate at the segment of entry and rostral but not caudal to it.

Tenderness induced by palpating the exercised muscle in DOMS might be conveyed in part by C-fibre afferents to the secondary neurons, since it is felt as diffuse and dull pain. In fact, there are C-fibre sensory receptors in the muscle that have nociceptive characteristics similar to cutaneous and visceral nociceptors, and that respond to various kinds of noxious stimuli (Kumazawa & Mizumura, 1977; Mense, 1977; Mense & Meyer, 1988). In addition, those receptors are sensitised to mechanical stimulation by inflammatory mediators such as bradykinin (Mense & Meyer, 1988). It has also been suggested that myelinated fibres are involved in DOMS (Weerakkody *et al.* 2001; Weerakkody *et al.* 2003). Although there has been dispute as to whether an inflammatory process is involved in the underlying mechanism of DOMS (Smith, 1991), the possibility can not be excluded that some inflammatory mediators or cytoplasmic components released as a result of the micro-injury in the muscle after eccentric contraction may sensitise the nociceptors to mechanical stimulation. Since the mechanism of hyperalgesia to mechanical stimulation in general is still poorly understood, electrophysiological studies of muscle nociceptor activities may shed some light on this field. Our preliminary experiment showed increased mechanical response and decreased mechanical threshold in C-fibres in this model (Taguchi *et al.* 2004). The present animal model would seem to be useful in investigating the mechanisms of DOMS and mechanism of mechanical hyperalgesia in general.

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Figure Legend

Figure 1. Schematic drawing of the experimental procedures.

(*A*) Methods of applying eccentric contraction to the extensor digitorum longus (EDL) muscle. One electrode was inserted transcutaneously near the sciatic nerve (+ pole) and the other near the common peroneal nerve (- pole). Repetitive contractions of the muscle were induced by cyclic electrical stimuli of the nerve. The hindpaw was fixed to a bar that was connected to the linearized motor, and it was pulled ('lengthened') synchronously with the muscle contraction so that the EDL muscle was stretched. Current intensity of the electrical stimulation was set at 3 times the threshold for twitch contraction, and frequency was set at 50 Hz to induce tetanic contraction. Contraction was repeated 500 times (1 sec contraction followed by 3 sec resting period). (*B*) Schedule of exercise and compression (upper panel), and the protocol of compression (lower panel) in the c-Fos experiment. Upper panel: On day 0 the animals underwent either eccentric contraction (ECC) or stretching of the muscle (SHAM). On day 2 the animals in the ECC and SHAM groups were each divided into 2 subgroups, one with and one without muscle compression. Lower panel: Exercised muscle (EDL muscle) was compressed with a force of 160 g by Randall Selitto apparatus for 10 s, and there was an interval of 20 s before the next compression. This session was repeated 60 times.

Figure 2. Decreased pain thresholds after ECC were found with Randall-Selitto test but not with von Frey hair test.

A, Withdrawal thresholds of the eccentrically exercised muscle measured by Randall-Selitto apparatus (n=6 for each group). Abscissa: days after exercise, ordinate:

withdrawal threshold. Closed circles show data from the ECC group, open circles show the result of the SHAM group. Randall-Selitto test showed decreased pain threshold after ECC. *$p<0.05$, ***$p<0.001$ compared with the value immediately before the day of exercise (-1 day). $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared with the SHAM group on each day after treatment. *B*, Withdrawal threshold for the skin over the exercised muscle measured by von Frey hairs (n=7 for each group). The presentation is the same as in *A*. No significant differences were found between groups on each day, or between -1 day and days after ECC (non-parametric Friedman test followed by Dunn's multiple comparison test). These results suggest that the pain threshold was decreased in the deeper tissue, probably in the muscle.

Figure 3. C-Fos-ir neurons in the ipsilateral dorsal horn of the spinal cord at L4.
A-D: Camera lucida drawings of representative sections from four groups 2 days after treatment: SHAM group (*A*), ECC group (*B*), SHAM+compression group (*C*), and ECC+compression group (*D*). Labelled neurons are represented as black dots. Note that the number of labelled neurons increased in the ECC+compression group on the second day. This was especially marked in the superficial dorsal horn. *E* and *F*: Photographs with different magnification of the same section as in *D*, showing c-Fos-ir neurons in the ipsilateral dorsal horn of the spinal cord at L4. Calibration bars: 100 μm in E and 50 μm in F, respectively.

Figure 4. Segmental distribution of the c-Fos-ir neurons in the dorsal horn of the spinal cord at L2-L6.

A: Representative camera lucida drawing of the c-Fos-ir neurons in the dorsal horn of the spinal cord at L2-L6 in a rat with ECC+compression. Labelled neurons are represented as black dots. The largest number of labelled neurons was observed in the superficial dorsal horn of L4 segment, and smaller numbers were seen also in L2 and L3 segments. *B*: Summary of segmental distribution of c-Fos-ir neurons in the spinal dorsal horn (L2-L6). Number of labelled neurons per section in the entire dorsal horn in each segment is shown. The number of animals used was 4 in SHAM, SHAM+compression, and ECC+compression groups, and 5 in ECC group. Note that there is a significant increase in the number of c-Fos-ir neurons at L4 in ECC+compression group compared with the other three groups (**$p<0.01$). The number of c-Fos-ir neurons at L2 and L3 in ECC+compression group tended to be greater, but differences among the four groups were not significant.

Figure 5. Number of c-Fos-ir neurons in the different areas of the spinal dorsal horn at L4 (*A*) and suppression of its increase by morphine (*B*).

In *A*, TTL (DH): total number of labelled neurons in the entire dorsal horn, SDH: superficial dorsal horn, PN: proprius nucleus, NDH: neck of the dorsal horn. The number of animals was 4 for SHAM, SHAM+compression, and ECC+compression groups, and 5 in ECC group. A significant increase in the number of c-Fos-ir neurons was observed in the superficial dorsal horn compared with each of other three groups (**$p<0.01$, ***$p<0.001$). The numbers of c-Fos-ir neurons in the proprius nucleus and the neck of the dorsal horn looked somewhat increased in the ECC+compression group, but the difference was not statistically significant. In *B*, black columns show the number of c-Fos-ir neurons per section in the animals that received morphine (10 mg/kg, i.p.) 20