Persistent deep mechanical hyperalgesia induced by repeated cold stress in rats

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Abstract

Chronic muscle pain of the neck, shoulder and low back is quite common and often related to a stressed condition. In this study we tried to make a model of long-lasting muscle mechanical hyperalgesia based on one type of stress, repeated cold stress (RCS) (Kita et al, Folia Pharmacol Jpn. 1975; 71: 195-210). We first validated a method of measuring the muscle mechanical nociceptive threshold through skin, with surface anesthesia of the skin covering the muscle. We found that a pressure test using a Randall-Selitto analgesiometer equipped with a larger probe (ϕ 2.6 mm) can measure the deep mechanical withdrawal threshold even under the presence of cutaneous punctuate hyperalgesia. RCS was performed by changing the temperature from 22 °C to either 4 °C (RCS at 4 °C) or -3 °C (RCS at -3 °C) every 30 min, and then maintained at 4 °C/-3°C from 17:30 to 10:00 the next day. RCS at 4 °C for 5 days induced bilateral deep mechanical hyperalgesia lasting 2-3 weeks without cutaneous punctuate hyperalgesia. Deep mechanical hyperalgesia observed after RCS at -3 °C lasted longer (~6 weeks) and was severer than RCS at 4 °C. Bilateral cutaneous punctuate hyperalgesia was also observed with RCS at -3 °C. Intramuscular injection of lidocaine confirmed that the muscle was hyperalgesic. RCS might serve as a useful model for study of the mechanism of chronic muscle pain and its treatment.

1. Introduction

Chronic pain of musculoskeletal structures in the neck, shoulder, low back and elsewhere is quite common. Persistent muscle pain has been studied in muscle inflammation models (Radhakrishnan et al.,2003; Reinert et al., 1998), but inflammation is seldom the cause of chronic muscle pain conditions; rather, they are often related to a stressed condition (Marras et al., 2000; Sato et al., 2002). In contrast stress has long been known to induce analgesia (stress-induced analgesia)(Bodnar et al., 1980; Lariviere and Melzack 2000). The recent literature, however, has shown that some forms of stress induce hyperalgesia (Imbe et al., 2006, for review). The relation between stress and muscle pain, though acute, was also shown in experimental conditions in humans (Bansevicius et al, 1997; Leistad et al, 2006). One experimentally stressed condition that is known to cause generalized mechanical hyperalgesia, is repeated cold stress (RCS, originally called specific alteration of rhythm in temperature (SART) stress) (Kita et al., 1975). In this form of stress mice and rats are transferred every 30 min (Satoh et al., 1992) or one hour (Kita et al, 1975) from a room at 22-24 °C to the cold room at 0 ~ 4 °C (mice) or 0 ~ -3 °C (rats). RCS is a condition somewhat similar to working in a cold storeroom, and persons who are working in such places often complain of musculoskeletal pain (Dovrat and Katz-Leurer, 2007; Pienimaki, 2002 for review of older papers). Despite such reports, the existence of muscular mechanical hyperalgesia has not yet been studied in RCS models.

A problem in examining muscle hyperalgesia in awake animals is that the skin and subcutaneous tissues intervene between the stimulation probe and the muscle to be

tested. Muscular mechanical hyperalgesia has been evaluated through skin in awake animals, but in such instances inflammation or other manipulation was introduced directly into the muscle (Skyba et al., 2005; Dina et al., 2008a). Use of larger probes has been recommended (Fischer 1987), but there have been claims of contamination from cutaneous sensation (Jensen et al, 1986: Kosek et al, 1999). In a previous experiment in healthy humans, we found that the pressure pain threshold measured with larger probes (ϕ > 2 mm) was not changed by surface anesthesia of the skin with lidocaine (Takahashi et al., 2005). Similar observation was reported by Graven-Nielsen et al. (2004). However, we do not know if use of a larger probe allows us to evaluate the muscle mechanical nociceptive threshold in rats, as well as in cases with cutaneous hyperalgesia. Therefore, we first examined which sizes of probe can be used to measure the muscle mechanical nociceptive threshold of normal rats, and validated this method in animals with muscular and cutaneous mechanical hyperalgesia after a single intramuscular injection of acid. Then, using this validated method, we examined whether RCS at different temperatures induced muscle mechanical hyperalgesia in rats.

Preliminary accounts of this experiment have been reported elsewhere (Mizumura et al., 2007; Nasu et al., 2007)

2. Methods

2-1. Animals

Sixty-four male Sprague-Dawley rats (200 g at the beginning of the experiment, Japan SLC) were used. The animals were housed two to three per cage under controlled temperature (22 ± 1°C) except during the period of RCS treatment and on a 12 h light/dark cycle, and had free access to food and water. When animals were exposed to RCS at -3°C, water contained in agar was given (500 g/5 days).

All experiments in the present study were conducted according to the Regulations for Animal Experiments in Nagoya University, the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions in Japan, and Ethical Guidelines of the International Association for the Study of Pain (Zimmermann et al., 1983).

2-2. RCS exposure

The RCS schedule was as follows: Rats were kept at 4 (RCS at 4 °C) or -3 °C (RCS at -3 °C) from 19.00 h on the first day to 10.00 h the following morning and then alternately exposed to room temperature (22 °C) and cold temperature (4 or -3 °C) at 30-minute intervals from 10.00 h to 17.30 h (Fig. 1, left). These procedures were repeated for 5 days.

Animals were divided into three groups (n = 8 for each group). The first group of animals was exposed to RCS at 4 °C (RCS at 4 °C group), the second group was exposed to RCS at -3 °C (RCS at -3 °C group), and the third group was kept in the RCS chamber, and moved between two compartments like the other two groups but without any temperature shifts (temperature was kept at 22 °C) and served as a control (Sham RCS group). They were housed two to three per metal-mesh cage (22 x 43 x 19 cm) with a sawdust-filled tray at the bottom, and were exposed to repeated temperature changes in a cold showcase that had two separate compartments with different temperatures (one at room temperature, the other either 4 °C or -3 °C), equipped with a moving shelf (automated RCS device constructed by the first author, Fig. 1, right). This shelf automatically transferred the caged rats over 30 sec at pre-set intervals from one compartment to the other. Possible disturbance to the sleep cycle of rats by transfer between compartments was minimized with this system. The rate of increase in body weight was slowed during the RCS period, but it recovered within a week after the end of the stress.

2-3. Withdrawal threshold measurement:

In the following measurements except probe size experiment, the experimenter was blind to which group an animal belonged. To attain this in RCS experiments, rats for other experiments were mixed with the RCS rats and measured at the same time.

2-3-1. Randall-Selitto test

A Randall-Selitto analgesimeter (Ugo Basile, Italy) was used to measure the withdrawal threshold of the deep tissues. The animals were restrained with a towel around the trunk to calm them, and treated gently during the experiments. A cone-shaped pusher with a rounded tip (tip diameter: 1.3 mm - commercially available, or 2.6 mm - made in our laboratory) was applied to the belly of the lower hind leg extensors including the extensor digitorum longus (EDL) muscle through shaved skin. The speed of applied force was set at 156.8 mN/s and the cut-off point was set at 2450 mN to avoid damaging the tissue. The intensity of the pressure that caused an escape reaction was defined as the withdrawal threshold. Training sessions were carried out for at least four consecutive days. Measurements were performed 9 times at about 90 sec intervals and the mean value of the last 5 trials was taken as the threshold.

2-3-2. von Frey hair test

The mechanical withdrawal threshold of the skin over the lower hind leg extensors and plantar surface was measured with self-made von Frey hairs (VFHs, diameter: 0.5 mm, bending forces 36.5-1756.8 mN in quasi-logarithmic order) because the mechanical strain induced by thin VFHs barely reaches the deeper muscle layer (Takahashi and Mizumura, 2004). The rats were restrained at the trunk with a towel, similar to in the Randall-Selitto test, and each filament was applied to the skin. Every filament was applied two times at intervals of a few seconds. The threshold was determined by the method of limits (briefly, changing the forces of VFHs up and down), and if an animal showed at least one withdrawal response, this was taken as a positive response.

2-3-3. Local anesthesia

In human studies 30 min application of EMLA cream (AstraZeneca Inc., UK) containing prilocaine 25 mg + lidocaine 25 mg in 1 g reportedly blocks nociceptive afferents up to 1 to 2 mm from the surface (Bjerring and Arendt-Nielsen, 1990). We therefore applied EMLA cream to the shaved skin over the lower hind leg extensors for 30 min in 10 rats and then removed it with ethanol. The contra-lateral side was used as the control receiving no EMLA treatment. Mechanical withdrawal threshold was measured 1 day before and shortly after the EMLA treatment with both VFHs and the Randall-Selitto apparatus equipped with one of two probes (one half of rats with 1.3mm probe, the rest with 2.6 mm probe). In the next week the same procedure but with different sized probe was repeated on the same group of rats (Fig. 2A). The VFH threshold measured on the day when Randall-Selitto measurement with 1.3 mm probe was done, was taken for the value of a rat. Measurement on the contralateral side was done after EMLA side measurement with the same procedure, except without EMLA cream treatment.

To be sure that the Randall-Selitto apparatus with a large probe measured deep mechanical withdrawal threshold in the presence of cutaneous hyperalgesia, we injected acid solution (physiological saline at pH 4.0, 20 μl) (to the same as the first injection in the method of Sluka et al., 2001) to the EDL muscle in fourteen rats under the guidance with electrical stimulation through a house-made injection needle to verify the exact location of the tip of the needle. In half of them (EMLA group) EMLA cream treatment was done as described above 3 hrs after acid injection, and 30 min

later the withdrawal threshold was measured again. In the remaining seven rats EMLA cream was not applied but the removal procedure with ethanol was done (control group).

To confirm that deep mechanical hyperalgesia after RCS was from muscle, lidocaine (AstraZeneca Inc., UK 30 μl) was injected into the EDL muscle (not into the tibialis anterior muscle to minimize diffusion of lidocaine to the skin), in 8 rats 4 days after RCS under guidance with electrical stimulation, as in the acid injection, and mechanical withdrawal threshold was measured before and 1 hour after injection. At least 2 hours elapsed between two measurements before and after the injection. For the control, 8 rats received saline (pH 7.6) injection 4 days after RCS with the same method.

2-4. Statistical analysis

The data from the Randall-Selitto test are presented as mean ± S.E.M. Two-way ANOVA with repeated measures was used, and post hoc comparisons was performed by Bonferroni's test. Each group in acid injection experiment was analyzed by one-way ANOVA followed by Bonferroni's test.

The VFH test data are presented as median and interquartile range (IQR). Results of surface anesthesia in normal animals were analyzed with Wilcoxon test, and those of acid treated animals, RCS groups and intramuscular lidocaine after RCS were analyzed by Friedman test followed by the Holm-Sidak method.

$P < 0.05$ was considered to indicate a significant change.

3. Results

I) Larger probe could measure deep mechanical withdrawal threshold even when the skin was hyperalgesic.

The VFH threshold of the skin over the EDL muscle was about 270.8 mN (IQR: 221.5-320.1 mN) in normal rats. After 30 min of EMLA cream treatment, the threshold was clearly and significantly increased to 765.7 mN (IQR: 667.9-1212.3 mN) (Fig. 2B, $p < 0.01$, Wilcoxon test). The Randall-Selitto threshold measured with a commercially supplied probe having a diameter 1.3 mm at the tip was 606.0 ± 190.7 mN before treatment. EMLA cream treatment had significant effect on the threshold measured with this probe ($F_{1, 18} = 46.30$, $p < 0.0001$) and there was significant side*treatment interaction ($F_{1,18} = 31.81$, $p < 0.0001$). The threshold of EMLA treated side was significantly increased after EMLA treatment (open circle in Fig. 2C left, $p < 0.001$, Bonferroni's test). The threshold of the untreated side (solid circle in Fig. 2C, left) remained unchanged ($p > 0.05$, Bonferroni's test). On the other hand, the Randall-Selitto threshold measured with a house-made probe, which had a diameter of 2.6 mm at the tip, was not altered at all by surface anesthesia (Fig. 2C, right). The threshold measured with this probe (891.7 ± 122.2 m N) was significantly higher than that measured with the probe of 1.3 mm (622.8 ± 178.2 mN, $p < 0.0001$, paired t test, $n = 10$).

Next we examined whether this method is also valid in the presence of cutaneous punctuate hyperalgesia. In this series we used only the larger probe for the Randall-Selitto test, because the value measured with a smaller probe (ϕ 1.3 mm) was

shown to be influenced by surface EMLA (described above), suggesting contamination from cutaneous sensibility. Hyperalgesia of both skin and muscle was induced by acid injection to the muscle. Acid solution (pH 4.0, 20 μl) was injected into the EDL muscle of both sides. The VFH threshold applied to the skin over the EDL muscle gradually decreased after acid injection (punctuate hyperalgesia) ($F_{2,12} = 11.543$, $p < 0.005$), and that at 3 hrs after injection was significantly decreased from the 'pre' value in the control group ($p < 0.001$ compared with 'pre' by Holm-Sidak method, Fig. 3A, left). In contrast, the decreasing VFH threshold was reversed and even became significantly higher than the 'pre'-injection value on the side treated with EMLA cream 3 hrs after acid injection ($F_{2,12} = 8.583$, $p < 0.001$, $p < 0.05$ compared with 'pre' by Holm-Sidak method, Fig. 3A, right). The Randall-Selitto threshold was decreased overall after acid injection (Fig. 3B, $F_{2,24} = 22.51$, $p < 0.001$), and there was no difference between treatment groups (EMLA and sham) ($F_{1, 12} = 0.1606$, $p > 0.05$). No significant interaction between time*treatment was detected ($F_{2,24} = 0.4668$, $p > 0.05$), therefore each group was analyzed separately. Unlike VFH threshold, the reduced Randall-Selitto threshold by acid was not reversed with EMLA cream treatment (n.s. compared with 1hr after acid injection; $p<0.01$ compared with 'pre, Bonferroni's test). These results suggest that VFHs with tip diameter of 0.5 mm measure mechanical withdrawal threshold of skin, while the Randall-Selitto apparatus with a probe of 2.6 mm in diameter measures that of deep tissues, possibly muscle, even when the skin is hyperalgesic. Therefore, we used the Randall-Selitto apparatus equipped with this large sized probe (2.6 mm) in the following experiments to measure the muscle mechanical withdrawal threshold.

II) RCS at 4 °C induced muscular mechanical hyperalgesia but not cutaneous

mechanical hyperalgesia

Before the days of RCS at 4 °C, the withdrawal threshold of the RCS group measured with the Randall Selitto apparatus was stable at 881.8 ± 96.3 mN ('pre 2' of the right side). A comparison of muscle withdrawal threshold among different temperature groups (sham RCS, RCA at 4 °C and at -3 °C groups. -3 °C group is not shown in Fig. 4) showed a significant difference ($F_{2,189} = 26.40$ for the right side and $F_{2,189} = 21.49$ for the left side, $p < 0.0001$ for both sides). Time* temperature interaction was also significant ($F_{18,189} = 4.080$ for the right side and $F_{18,189} = 4.398$ for the left side, $p < 0.0001$ for both sides). There was a significant difference between the RCS at 4 °C group and the sham RCS group ($p < 0.01$ by post hoc analysis by Bonferroni's test in Fig. 4, left). The sham RCS group showed no significant change during the observation period of 42 days (Fig. 4A and B, open circle in left graphs), but the threshold decreased bilaterally in RCS at 4 °C group (Fig. 4A and B, solid circle in left graphs) to 640.4 ± 151.5 mN (right side) and 594.4±64.7 mN (left side) 1 day after RCS. Statistically significant decrease compared with 'pre 2' lasted up to 14-21 days after RCS with partial, transient recovery 7 days after RCS. Complete recovery was observed 21-28 days after RCS (Fig. 4A and B, left graphs).

In contrast to the Randall-Selitto threshold, the threshold measured with VFH was not different between the RCS at 4 °C and sham RCS groups, and did not change at all on either side in the RCS group (Right side: $\chi^2 = 12.045$, df = 9, p = 0.211, Left side: $\chi^2 = 12.122$, df = 9, P = 0.207, Friedman test, Fig. 4A, B, right graphs).

III) Lower temperature RCS (-3 °C) prolonged muscular hyperalgesia and induced cutaneous mechanical hyperalgesia

The Randall-Selitto threshold of the RCS at -3 °C group differed from the sham RCS ($p < 0.001$, Bonferroni's test, F values are presented in the previous section). It was also different from the RCS 4 °C group ($p < 0.001$, Bonferroni's test), and the decrease in the threshold in legs of both sides was much stronger, and lasted for a longer period than that induced by RCS at 4°C: The lowest withdrawal threshold was 465.3 ± 58.7 mN on 21 days after RCS in the right side and 438.6 ± 41.0 mN on 14 days after RCS in the left. Statistically significant reduction of withdrawal threshold from 'pre 2' was observed up to 35-42 days (Fig. 5, left graphs). We continued observation up to 56 days after RCS and confirmed complete recovery on 49 days after RCS (data not shown).

Different from RCS at 4 °C, RCS at -3 °C clearly and significantly reduced VFH threshold (punctuate hyperalgesia) in the lateral lower leg (right side: $F_{9,63} = 11.74$, left side: $F9,63 = 8.083$, $p < 0.001$ for both sides), and the significant decrease from 'pre 2' value lasted up to 14-21 days after RCS (Fig. 5, right graphs). This decrease was seen on both sides of the body.

To ascertain that the deep tissue that was hyperalgesic after RCS was muscle, we injected lidocaine into the EDL muscle 4 days after RCS at -3 °C and examined changes in withdrawal threshold. As seen in Fig. 6B, the Randall-Selitto threshold was

significantly changed along time ($F_{2,28} = 43.27$, $p < 0.0001$), and there was a significant time*treatment (saline or lidocaine) interaction ($F_{2,28} = 6.798$, $p < 0.005$). The threshold clearly decreased 4 days after RCS in both treatment groups ('pre injection', $p < 0.001$ compared with 'pre RCS' by Bonferroni's post hoc test), and this decrease was significantly reversed by intramuscular injection of lidocaine ('post injection', $p < 0.001$ compared with the 'pre injection', by Bonferroni's post hoc test). In contrast, saline injection had no effect on decreased Randall-Selitto threshold ('post injection', $p > 0.05$ compared with 'pre injection' and $p < 0.001$ compared with 'pre RCS'). VFH threshold was also significantly changed in both the saline and lidocaine injection groups ($F_{2,14} = 10.604$, $p < 0.002$ for the lidocaine group, $F_{2,14} = 32.962$, $p < 0.001$ for the saline group) . Decreased VFH threshold by RCS in the lateral lower leg ('pre injection', *$p < 0.05$ compared with 'pre RCS') was not influenced by injection of lidocaine ('post injection', n.s. $p > 0.05$ compared with 'pre injection', Fig. 6A), demonstrating that intramuscular lidocaine did not reach the cutaneous structure.

4. Discussion

The present experiment demonstrated, for the first time, the existence of deep (muscular) mechanical hyperalgesia lasting longer than 3 weeks after RCS. Muscular mechanical hyperalgesia was longer and severer after RCS at -3 °C than RCS at 4 °C. Cutaneous punctuate hyperalgesia was not observed after RCS at 4 °C, but was observed after RCS at -3 °C.

1) Evaluation of deep mechanical hyperalgesia

Use of a large probe such as $\phi > 5$ mm has been recommended for muscle mechanical pain threshold measurement in humans, and probes with surface area 1 cm^2 (diameter 1.1 cm) are often used in clinical settings (Fischer et al., 1987), although the rationale for this has not been clarified. Behavioral evaluation of muscular nociceptive threshold has been made in animals (Radhakrishnan et al., 2003; Schafers et al., 2003; Dina et al., 2008a, b) but only in conditions where cytokines or inflammation-inducing substances were directly injected into the muscle and where muscular hyperalgesia was highly expected. In contrast to these experiments, we have proven by means of surface anesthesia that the larger probe (ϕ 2.6 mm) measures deep mechanical nociceptive threshold even when cutaneous punctuate hyperalgesia exists. Additionally, we confirmed with intramuscular injection of lidocaine that the deep tissue that was hyperalgesic after RCS was muscle. This result not only confirms human studies by us (Takahashi et al., 2005) and Graven-Nielsen et al. (2004), but shows the robust usefulness of a larger probe in measuring mechanical withdrawal threshold in deep

structures.

The reason a larger probe (ϕ 2.6 mm in this experiment) can measure deep mechanical threshold must be considered. Our preliminary result from a computer simulation of stress transmission through skin to muscle using a 3-dimensional finite element model showed that stress applied through the skin was localized in the surface structure when the probe size was small, e.g. ϕ 0.5 mm (size of von Frey filament used in the present experiment), but was transmitted to deeper tissues as far as muscles with larger probes (Takahashi and Mizumura, 2004). This computer simulation also suggested that the transmission ratio to the muscle could be influenced by the characteristics of intervening structures, e.g. thickness of subcutaneous fat. Although stress transmission is improved by use of larger probes, we do not know why cutaneous pain is not induced by this method. One reason might be a higher mechanical threshold of cutaneous C-fiber afferents (Suzuki et al., 2002) than muscle ones (Taguchi et al, 2005). Another reason might be lateral inhibition based on higher innervation density in the skin. However, this remains speculation, and we must be aware of the limitations of this method of deep nociceptive threshold measurement. As far as rats of size 200 g-370 g used in this experiment, the validity of this method was demonstrated by the present results.

Another possible factor limiting usage of this method would be severity of cutaneous hyperalgesia. We induced mild cutaneous hyperalgesia by single acid injection, and found that this level of cutaneous hyperalgesia did not influence the deep nociceptive threshold. On the other hand, Polianskis et al. (2002b) reported deep pressure pain

threshold measured by pressure–cuff algometry, which was not influenced by surface anesthesia in normal condition, decreased after capsaicin cream treatment, suggesting that cutaneous hyperalgesia influenced deep pressure pain threshold. This result suggests that when cutaneous hyperalgesia is severe, the deep pressure pain (nociceptive) threshold measured with pressure-cuff algometry was influenced, and that the Randall-Selitto threshold measured with a larger probe could be influenced as well. Therefore, in such instances injection of an anesthetic into the muscle or surface anesthesia is necessary to assure the existence of deep mechanical hyperalgesia.

The threshold force measured with the probe of 2.6 mm in diameter was significantly higher than that measured with the probe of 1.3 mm in diameter after EMLA treatment (the latter is considered to be also the deep nociceptive threshold). However, when compared by pressure level, the threshold measured with 2.6 mm probe was lower. This might have resulted from the fact that the stress transmission is improved with a larger probe, as discussed in the previous section. It is well known that the larger the probe is, the lower is the sensation threshold in terms of pressure (Jensen et al, 1986; Estebe et al, 2000; Polianskis et al, 2002a). Spatial summation might be another factor inducing the threshold difference.

2) Muscular mechanical hyperalgesia by RCS

This is the first report to show the existence of muscle mechanical hyperalgesia after RCS. In previous reports on mechanical hyperalgesia by RCS in rats and mice, the same Randall-Selitto analgesimeter was used as we used in this study, but with a different

shaped probe from ours, namely a plate-like or wedge-like probe (Satoh et al., 1992). In addition, the probe was applied to the tail or to the dorsal hindpaw (Hata et al., 1988a; Satoh et al., 1992) which are not muscular structures.

Until recently there has been only one model (acid injection model) other than inflammation in which long lasting muscle mechanical hyperalgesia may exist (Yokoyama et al., 2007). Dina et. al. (2008a, b) have also recently reported that IL-6 priming will provide a condition that may induce long-lasting muscle hyperalgesia. The RCS model will serve as another unique model for study of the mechanisms of chronic muscle mechanical hyperalgesia. Continuous cold did not induce some of the changes induced by RCS (Hata et al., 1988b); therefore, repetitive alteration of the temperature in a short period of time (30 min-1 hr) is important for the effect of RCS.

The effect of RCS was quite different from cold-warm treatment to alleviate pain and improve circulation in the extremities. The latter treatment is limited to one of the extremities or part of it, whereas in RCS the entire body is exposed to temperature change. Therefore, autonomic functions must be more profoundly influenced by RCS than by localized cold-warm treatment. In addition, stress response must be stronger in RCS. These factors might have resulted in the different effects.

This model has behavioral signs of depression/anxiety (Hata et al., 1995, 1999, 2001), autonomic dysfunction (Kita et al., 1975) and decreased concentration of serotonin in CSF (Hata et al., 1991). Thus, RCS model has some similarities with human fibromyalgia syndrome, which is characterized by generalized hyperalgesia, existence

of more than 11 tender points, a depressed/anxiety state in many cases, irritable bowel syndrome, sleep disturbance, and low serum serotonin level (Russell et al., 2006). The present results, by demonstrating the existence of muscle mechanical hyperalgesia, further support the usefulness of this model for the study of chronic muscle pain such as fibromyalgia.

3) More profound effect of RCS at -3 °C

RCS at 4 °C induced bilateral muscular mechanical hyperalgesia only (decreased Randall-Selitto threshold), but RCS at -3 °C additionally induced cutaneous punctate hyperalgesia (decreased VFH threshold) of both sides. One possible, though not likely, mechanism for this different effect of cold on cutaneous and muscle mechanical nociception might be related to the difference in tissue hypoxia in both tissues. In cold environments, muscle tonus must be continuously increased to enhance heat production, and vasoconstriction surely occurs to protect against heat loss, resulting in relative hypoxia in the muscle. As it has been reported that ischemia induces increased mechanical sensitivity in muscle thin-fiber afferents (Kaufman et al., 1984; Kaufman et al., 1988), relative muscle hypoxia during the cold exposure periods of RCS might provide a condition for increased mechanical sensitivity of muscle nociceptors. Cutaneous nociceptors are reportedly also sensitized to mechanical stimulation by acidic pH which occurs in ischemic tissues (Steen et al., 1992). However, oxygen consumption in the skin is low relative to contracting muscle. Therefore, stronger vasoconstriction induced by -3 °C might have resulted in hypoxia in the skin severe enough to sensitize nociceptors, so that cutaneous mechanical hyperalgesia could have occurred only by

RCS at -3 °C. Because the whole body was exposed to temperature change, generalized hyperalgesia could have been induced despite the peripheral origin. This peripheral mechanism might be able to explain the mechanical hyperalgesia during the period of RCS application (reported in Ohara et al, 1991), but it would be difficult to assume that it still makes a contribution to the mechanical hyperalgesia long after the end of RCS. If there are another peripheral factors inducing mechanical hyperalgesia is to be studied.

Alternatively, and more likely, a central mechanism might be the cause for the different effect of RCS on skin and muscle nociception. Hata's group has reported that the descending inhibitory system is impaired after RCS (Hata et al., 1991). Notably, Yu and Mense (1990) have reported that the effect of descending inhibition is stronger on muscle nociception than on the cutaneous nociception. Considering these findings, it may be hypothesized that impairment of the descending inhibitory system by RCS at 4 °C might be weaker than that by RCS at -3 °C, so that while muscle hyperalgesia results after RCS at 4 °C, both cutaneous and muscle hyperalgesia result after RCS at -3 °C. Sluka's group reported that activation of the descending facilitatory system from RVM is involved in muscle hyperalgesia induced by repeated acid injections (Tillu et al., 2008). Therefore, activation of this system might also be involved in the muscle mechanical hyperalgesia after RCS, but whether muscle nociception is more profoundly influenced by this system remains to be clarified. At the spinal level, involvement of glutamate through NMDA receptors, substance P, and CGRP has been reported in RCS induced hyperalgesia (Satoh et al., 1992; Kuraishi et al., 1993; Okano et al., 1995). Impairment of the opioid system was also reported in RCS treated rats (Omiya et al., 2000), and a recent proteomics study showed changed expression of

neurotransmission-related substances in the mesencephalon and cerebellum (Fujisawa et al., 2008). However, it is not known whether these changes differ between cutaneous and deep nociceptive systems.

The present experiment showed that a larger probe can measure deep nociceptive threshold even in a condition with cutaneous hyperalgesia, providing a rationale for use of a larger probe for the measurement of deep (muscle) mechanical withdrawal threshold. This will make evaluation of muscle nociception in awake animals easier, and will promote the study of muscle pain. The present experiment also showed that RCS at 4 °C induced prolonged muscle mechanical hyperalgesia, whereas RCS at -3 °C additionally induced cutaneous mechanical hyperalgesia. We propose that this different effect is explained by different descending controls on muscle and cutaneous nociception. This model will be useful not only for the study of the chronic muscle pain mechanism, but also for the study of the descending control system.

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Figure Legend

Figure 1. House-made automated device for loading repeated cold stress (RCS), and exposure schedule

The device (right figure) is composed of two compartments, an upper compartment with cold (4 or -3 °C) temperature and a lower one with room temperature. For five days, rats in mesh cages are automatically moved over 30 sec from one compartment to the other every 30 min during daytime, and kept in the cold compartment during the night as shown in the schedule (left).

Figure 2. The absence of a surface anesthesia effect on the withdrawal threshold measured with the Randall-Selitto analgesiometer equipped with a larger probe

A: Schedule for testing withdrawal threshold with EMLA cream treatment. Measurement was done twice for each rat with an interval of week. Measurement was done the first week with VFH then the Randall-Selitto test with one of the two probes, and the following week with VFH then the Randall-Selitto test with the other probe. B: Change caused by EMLA cream in withdrawal threshold measured with VFHs. Data are presented as box (median ± interquartile range (IQR)) and whiskers (10 and 90 percentile values). Vertical axis: withdrawal threshold in mN (log scale), ** $p < 0.01$, Wilcoxon signed rank test compared with the value measured immediately before the EMLA cream treatment. C: Change caused by EMLA cream in withdrawal threshold measured with the Randall-Selitto analgesiometer (RS). Left: measured with a

commercially available probe (ϕ 1.3 mm), right: measured with a self-made larger probe (ϕ 2.6 mm). Data are presented as mean ± S.E.M. Open circles: anesthetized side (n = 10), filled circles: contralateral side (n = 10). *** $p < 0.001$, Bonferroni's test after two-way ANOVA with repeated measures. After 30 min of surface anesthesia, the withdrawal threshold measured with VFHs and that measured with the Randall-Selitto apparatus equipped with a commercially available probe increased, whereas that measured with the Randall-Selitto apparatus equipped with a larger probe did not change.

Figure 3. Withdrawal threshold measured by Randall-Selitto apparatus equipped with a larger probe was not influenced by surface anesthesia even in the presence of cutaneous hyperalgesia

A: Change in VFH threshold after acid injection and EMLA treatment. Left: control group (n = 7), right: EMLA group (n = 7). Acid was injected at the time shown with a solid arrow and EMLA cream or sham treatment at a fine or coarse broken arrow. Data are presented as box (median ± interquartile range (IQR)) and whiskers (10 and 90 percentile values). Vertical axis: withdrawal threshold in mN.* $p < 0.05$ and *** $p < 0.001$ compared with the value before injection ('pre') (Friedman test followed by Holm-Sidak test). B: Change in the withdrawal threshold measured with the Randall-Selitto analgesiometer equipped with a larger probe after acid injection (solid arrow), and EMLA cream (fine broken arrow) or sham treatment (coarse broken arrow). Data are presented as mean ± S.E.M. Open circles: anesthetized group with EMLA (n = 7), filled circles: control group (n = 7) wiped with ethanol. n.s. not significantly

different compared between EMLA and sham treated groups (two-way ANOVA with repeated measures), and compared between 1h and 3h after acid injection (Bonferroni's test. *$p < 0.05$, **$p < 0.01$ compared with 'pre' (Bonferroni's test).

Figure 4. RCS at 4 ºC induced bilateral mechanical hyperalgesia revealed by the Randall-Selitto method

Change in the withdrawal threshold after RCS in the right (A) and left hind leg (B). Left: Randall-Selitto threshold. Vertical axis: withdrawal threshold in mN, Horizontal axis: days after RCS (not linear in graphs in the right). Open circles: sham RCS group (n = 8), solid circles: RCS at 4 °C group (n = 8). Period marked with shadow: days of RCS or sham exposure. Mean ± S.E.M. ## $p < 0.01$ compared with the sham RCS group (Bonferroni's multiple comparison test following two-way ANOVA with repeated measures of three RCS groups, comparison between RCS -3 °C group is not shown). ** $p < 0.01$, *** $p < 0.001$ compared with the value 1 day before RCS ('pre 2') (by Bonferroni's multiple comparison test following two-way ANOVA with repeated measures). There was a significant difference between the sham RCS and RCS at 4 ºC group, and the sham RCS group showed no significant change during the entire observation period. The RCS at 4 °C group had decreased Randall-Selitto threshold up to 14 or 21 days after RCS. Right: VFH threshold of RCS at 4 °C group. Data are presented as box (median ± interquartile range (IQR)) and whiskers (10 and 90 percentile values). Vertical and horizontal axes are similar to the left graphs. The VFH

threshold of the lower leg skin of both sides showed no significant change during the entire observation period of 56 days (Friedman test followed by Holm-Sidak method). The same was true in the sham group (data not shown).

Figure 5. RCS at -3 ºC induced bilateral mechanical hyperalgesia revealed both by the Randall-Selitto method and the von Frey method

Change in the withdrawal threshold after RCS in the right (A) and left hind leg (B). The way of presentation is similar to Fig. 4 except solid circles represent data of RCS at -3 °C group. Left: Randall-Selitto threshold. n = 8 for both groups. ### $p < 0.001$ compared with the sham group (Bonferroni's multiple comparison test after two-way ANOVA with repeated measures) and the RCS at 4 °C group (not shown). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the value 1 day before RCS (‘pre 2’) (by Bonferroni's multiple comparison test after two-way ANOVA with repeated measures). Right: VFH threshold of RCS group. The VFH threshold of the lower leg skin of both sides showed significant change up to 14-21 days after RCS (* $p < 0.05$, ** $p < 0.01$ compared with ‘pre 2’, Friedman test followed by Holm-Sidak method). It must be noted that deep mechanical hyperalgesia lasted longer than RCS at 4 °C; in addition, cutaneous hyperalgesia was induced RCS at -3 °C.

Figure 6. Intramuscular injection of lidocaine increased Randall-Selitto threshold but not von Frey hair threshold that were both decreased after RCS

A: Change in VFH threshold at the lateral lower leg by RCS at -3 °C and intramuscular

lidocaine. Sham group (left) received intramuscular injection of saline (course broken arrow), and test group (right) received intramuscular injection of lidocaine (fine broken arrow) 4 days after RCS at -3 °C. n = 8 in both groups. * $p < 0.05$ compared with 'pre RCS', n.s. not significantly different from 'pre injection' (Friedman test followed by Holm-Sidak method). It is noted that with RCS -3 °C the VFH threshold was clearly decreased ('pre injection'), and this was not influenced by intramuscular injection of lidocaine ('post injection'), or by saline. B: Change in Randall-Selitto threshold by RCS and intramuscular injection of lidocaine (closed circle) or saline (open circle) in the same groups of animals in A. *** $p < 0.001$ and (n.s.) not significant compared with 'pre RCS', ### $p < 0.001$ and n.s. not significant compared with 'pre injection' (two-way ANOVA with repeated measures followed by Bonferroni's test). Note that intramuscular lidocaine clearly reversed deep mechanical hyperalgesia induced by RCS, confirming that deep mechanical hyperalgesia was from the muscle.