Article

Altered gene expression of RNF34 and PACAP possibly involved in mechanism of exercise-induced analgesia for neuropathic pain in rats

Shintaro Yamaoka[1], Yusuke Oshima[2,3,*], Hideki Horiuchi[4], Tadao Morino[1], Masayuki Hino[1],

Hiromasa Miura[1], Tadanori Ogata[1]

[1] Department of Bone and Joint Surgery, Ehime University Graduate School of Medicine, Shitsukawa, Toon, Ehime, 791-0295, Japan; E-Mail: shinyama@m.ehime-u.ac.jp (S.Y.); hino@m.ehime-u.ac.jp (M.H.) ; morino@m.ehime-u.ac.jp (T.M.); miura@m.ehime-u.ac.jp (H.M.) ogata@m.ehime-u.ac.jp (T.O.)
[2] Translational Research Center, Ehime University Hospital, Shitsukawa, Toon, Ehime, 791-0295, Japan; E-Mails: oshima-ehm@umin.ac.jp (Y.O.)
[3] Division of Bio-imaging, Proteo-Science Center, Ehime University, Shitsukawa Toon city, Ehime, 791-0295, Japan
[4] Ozu Municipal Hospital, Nishiozu, Ozu, Ehime, 795-8501, Japan; E-Mail: horiuchiehime@gmail.com (H.H.)
* Correspondence: oshima-ehm@umin.ac.jp; Tel.: +81-89-960-5343; Fax: +81-89-960-5346.

Abstract: Despite the availability of several modalities of treatment, including surgery, pharmacological agents, and nerve blocks, neuropathic pain is often unresponsive and sometimes progresses to intractable chronic pain. Although exercise therapy is a candidate for treatment of neuropathic pain, the mechanism underlying its efficacy has not been elucidated. To clarify the molecular mechanism for pain relief induced by exercise, we measured *Rnf34* and *Pacap* mRNA levels in the spinal cord dorsal horn of SNL rats, a model of neuropathic pain. SNL model rats exhibited stable mechanical hyperalgesia at least for 6 weeks. When the rats were forced to exercise on a treadmill, mechanical and thermal hyperalgesia were significantly ameliorated compared with the non-exercise group. Accordingly, gene expression level of *Rnf34* and *Pacap* were also significantly altered in the time course analysis after surgery. These results suggest that exercise therapy possibly involves pain relief in SNL rats by suppressing *Rnf34* and *Pacap* expression in the spinal cord.

1. Introduction

Many patients in clinics complain of pain in the trunk or extremities. In the field of spine surgery, there are many causes of neuropathic pain, such as traumatic injury, entrapment and compression syndrome and neoplastic disease. Neuropathic pain, which results from mechanical compression or degeneration of nervous tissue, is associated with allodynia, hyperalgesia, and continuous spontaneous pain. Accordingly, treatment of pain is one of the most important topics for clinicians in orthopedic surgery and anesthesiology. Several therapeutic modalities, including decompression surgery, pharmacological treatment, and nerve block, have been applied for the treatment of neuropathic pain. An operative treatment for lumber disc herniation has consistent evidence that short-term efficacy of surgery is higher than that of conservative treatment, but the long-term efficacy is not significant in compared to conservative treatment [1], thus surgical treatments are not always effective for neuropathic

pain. Regarding those conservative treatments, non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used for the treatment of musculoskeletal pain, but because these drugs act by inhibiting prostaglandin production, they are effective only for treatment of inflammatory pain. Typical pharmacological agents used to treat neuropathic pain include opioid receptor agonists, Ca-channel blockers, and monoamine re-uptake inhibitors. Opioid receptor (OR) and monoamine systems are the primary mechanisms that inhibit transmission of pain sensation. Accordingly, endogenous opioid receptor agonists are very powerful analgesic mechanisms. In the spinal cord, δ OR play important roles in antinociception [2]. Clinically, μOR agonists such as morphine are commonly used for the treatment of serious pain, including postoperative pain. Monoamines, such as noradrenalin [3] and serotonin [4], are also strong endogenous pain-relieving agents. Monoamines inhibit pain signals by the activating GABA signals [5]. Elevation of monoamines by re-uptake inhibitors has been used for pain relief care in patients with several neuropathic pain [6]. In addition, we reported previously that serotonin reuptake inhibitors ameliorated neuropathic pain induced by spinal cord injury [7]. Pregabalin, a structural analog of gamma-aminobutyric acid (GABA) that selectively binds the alpha2-delta (α2-δ) subunit of voltage-dependent calcium channels, possesses analgesic, anxiolytic, and antiepileptic properties. Gabapentin and pregabalin are regarded as first-line treatments for peripheral pain with a neuropathic component [8]. However pharmacological treatment also can not cure completely all symptoms of neuropathic pain.

Despite the availability of these pharmacological treatments and other conservative treatments such as physical therapy, many patients have intractable chronic neuropathic pain. Actually, to overcome neuropathic pain, we should propose a combination of those treatments. We focused on exercise therapy as an effective candidate of conservative treatment for neuropathic pain. Exercise therapy has been established as the main conservative therapy for patients with chronic lower-back pain [9], and there are few reports indicating that exercise therapy is effective for the treatment of sciatica in human[10]. On the other hand, in animal there are some reports. Stagg et al. reported that exercise training reversed thermal and tactile hypersensitivity in the rat SNL model. In addition, they found that exercise increased β-endorphin and met-enkephalin content in the rostral ventromedial medulla and the mid-brain periaqueductal gray area [11]. The forced exercise training also improved neuropathic pain after spinal cord injury in rats [12]. Leung et al reported that physical activity alters macrophage phenotype to increase IL-10 and prevent chronic pain in C57BL/6J mice [13]. Bobinski et al demonstrated that the exercise suppresses pain-like behaviors in animals with neuropathic pain by enhancing brainstem serotonin (5-HT) neurotransmission [14]. In neuropathic pain rat model, exercise induced analgesia could be mediated by desensitization of central μOR by endogenous opioids [15]. However, recent reports have not fully explained the underlying mechanism by which exercise therapy ameliorates neuropathic pain.

In this study, to clarify the mechanism of the effect of exercise on neuropathic pain due to nerve compression, we subjected SNL rats to enforced exercise on a treadmill and observed the molecular changes in the dorsal horn of the spinal cord.

2. Results

First, in order to confirm that ligation of the L5 spinal nerve (SNL model) caused neuropathic pain without deficiency of motor function, we observed hind-limb motor function using the BBB scale. All SNL animals exhibited full hind limb function (BBB score of 21) during the experimental period (data not shown). Pain-like behavior was assessed by mechanical stimulation using the von Frey filament test.

Figure 1 shows the pain threshold in response to mechanical stimulus in the ipsilateral and contralateral hindlimbs in the control group. In animals received SNL, the pain threshold decreased 3 days after surgery on both the ipsilateral and contralateral sides, and reached a plateau that was sustained until 6 weeks after surgery. After the operation, the pain threshold was significantly lower on the ipsilateral side than on the contralateral side. In the sham operation group, no significant change in pain threshold was observed after surgery (data not shown).
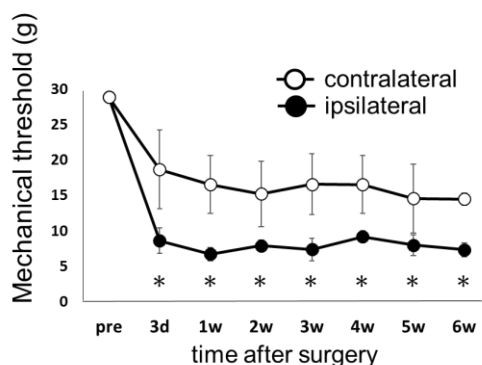
Figure 1. Time course of withdrawal latency in response to mechanical stimulation in the spinal nerve ligation (SNL) model without exercise (the control group). The hind paw withdrawal threshold by mechanical stimulation was determined by von Frey test. Measurements were performed before surgery (pre), and weekly for 6 weeks after surgery (1w, 2w, 3w, 4w, 5w, and 6w). Closed circles (●) represent the side ipsilateral to ligation, and open circles (○) represent the side contralateral to ligation. Data represent averages ± S.D. (n = 6) * p < 0.05 according to Wilcoxon signed-rank test.

Next, we assessed the behavioral effect of exercise on neuropathic pain (Fig. 2A). For this purpose, we divided the SNL rats into two groups, exercise and control (i.e., non-exercise).

In the behavior test, the pain threshold decreased 3 days after surgery in both the exercise and the control groups. In the exercise group, pain-like behavior improved starting 3 weeks after surgery. From 3 to 6 weeks after surgery, the pain thresholds were significantly higher (P < 0.05) in the exercise group than in the control group.
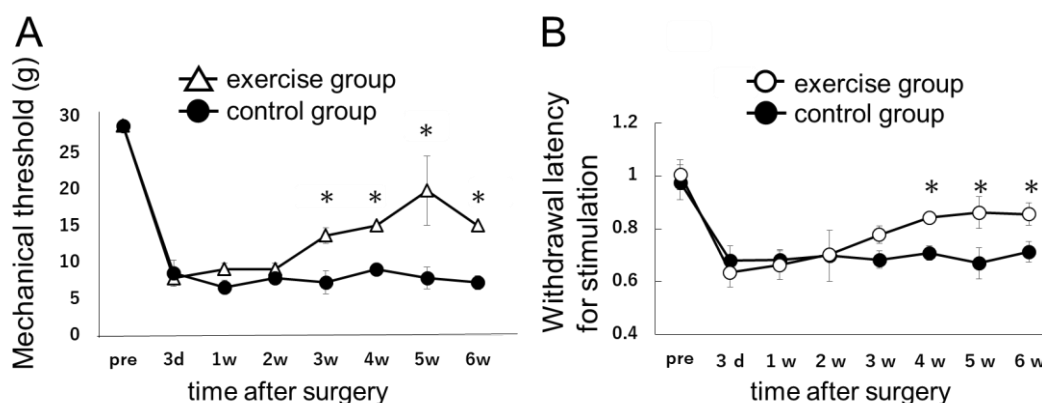
Figure 2. (A) Effect of exercise training on mechanical hyperalgesia in the hind paw ipsilateral to ligation in the SNL model. The mechanical threshold in the hind paw ipsilateral to ligation was compared between SNL model rats subjected to (exercise group) or not subjected to (control group) forced treadmill running. Open triangles (Δ) represents the exercise group, and closed circles (●) represents the control group. Data represent averages ± S.D. (n = 6) *p < 0.05 according to Wilcoxon

119   signed-rank test. Starting 3 weeks after the surgery, exercise training significantly improved
120   neuropathic pain induced by SNL. (B) The time course of withdrawal latency for thermal stimulation.
121   Open circles (○) represent the exercise group, and closed circles (●) represent the control group. The
122   withdrawal latencies for thermal stimulation are presented as the ratio of ipsilateral to contralateral.
123   Data represent averages ± S.D. (n = 6) *p < 0.05 according to Wilcoxon signed-rank test. Starting 3
124   weeks after the surgery, exercise training gradually improved neuropathic pain induced by SNL.
125   Four weeks after surgery or later, the withdrawal latency of exercise group was larger than that of
126   control group.

127   Figure 3 depicts the experimental procedure from the L5 spinal nerve ligation surgery to total
128   RNA extraction.
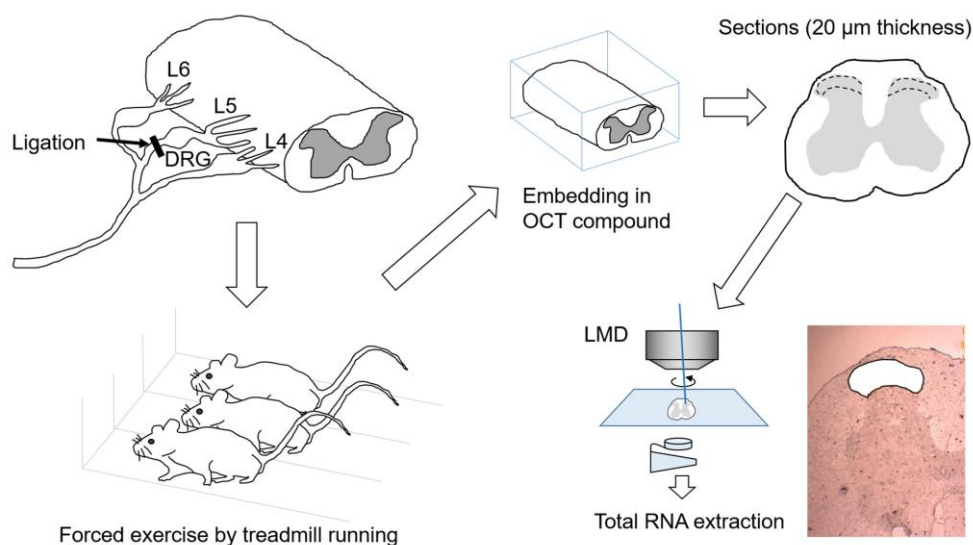
130   **Figure 3.** Schematic illustration of experimental procedures. L5 spinal nerve was ligated unilaterally
131   (on the right side) distal to the L5 dorsal root ganglion (DRG). SNL rats were subjected to forced
132   exercise on a treadmill as a model of exercise therapy. Spinal cord was resected at the T11 level and
133   embedded in OTC compound, and then 20-μm frozen sections were produced. The tissue from
134   laminae I–II of the dorsal horn was collected using a laser microdissection method, and the samples
135   were used for extraction of total RNA.

136   To elucidate how exercise therapy ameliorates neuropathic pain, we monitored differential
137   gene expression using a next-generation sequencing (NGS) method, RNA-seq. After the exercise
138   program was complete, we resected the spinal cord tissue and performed gene-expression analyses
139   by RNA-seq and quantitative RT-PCR, as shown in Fig. 4A. RNA-seq yielded about 6,000,000 reads
140   from total RNA samples derived from laminae I–II of the dorsal horn of the spinal cord. Of 17314
141   genes identified by mapping onto the rat genome, 499 exhibited significant differences in the
142   expression between the exercise and control groups. Specifically, 241 genes were upregulated in the
143   exercise group, and 258 were downregulated (Fig. 4B). Next, we performed tissue-specific functional
144   annotation focusing on the central nerve system, brain, and spinal cord. Of the differentially
145   expressed genes, 17 upregulated and 49 downregulated genes are thought to be mainly expressed in
146   brain and spinal cord (data not shown). Ultimately, we focused on *Rnf34* and *Pacap*, which were
147   significantly downregulated in the exercise group and are thought to be associated with pain.
148
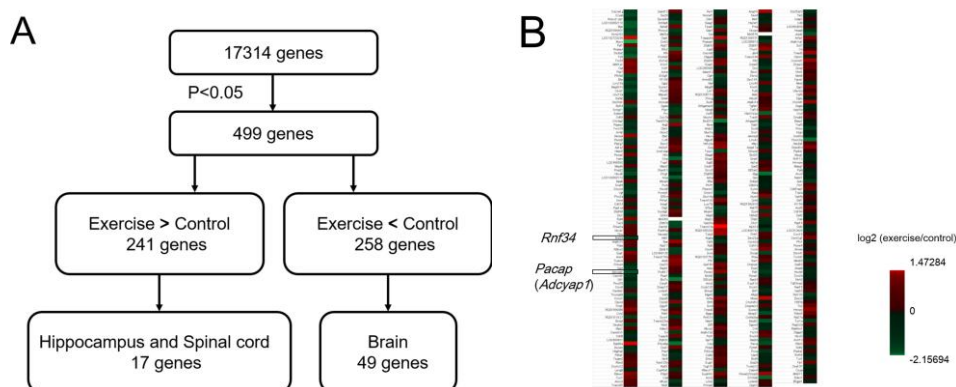
Figure 4. (A) Analytical flowchart of gene-expression analyses by RNA-seq, bioinformatics, and quantitative PCR. RNA samples isolated from dissected tissue from the exercise and control (non-exercise) groups 6 weeks after surgery were subjected to RNA-seq. In total, 17314 genes were initially identified. Those genes were sorted by relative expression level (exercise vs. control). In the exercise group, 241 genes were up-regulated and 258 genes were down-regulated with statistical significance (p < 0.05 according to Student's t-test). Those genes were annotated based on the tissue specificity of their expression. (B) Heat map of the gene expression profile obtained from the result of RNA-seq. Total of 499 genes (241 upregulated genes and 258 downregulated genes) were identified with statistical significance. *Rnf34* and *Pacap* (*Adcyap1*) were selected from the list of downregulated genes by tissue specific functional annotation. The fold change values (log2[exercise/control]) of *Rnf34* and *Pacap* were -0.72 and -1.01, respectively.

Next, we performed quantitative RT-PCR on *Rnf34* and *Pacap* and compared their levels between the exercise and control groups at 1, 3, and 6 weeks after surgery. Expression of *Rnf34* was significantly downregulated 3 weeks after surgery in the exercise group, but no significant difference between the exercise group and the control group was detected at 1 or 6 weeks after surgery (Fig. 5).
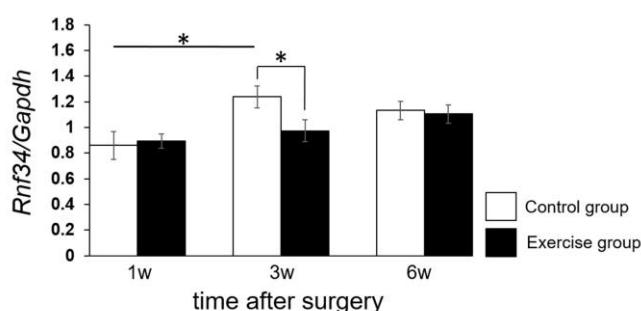
Figure 5. Effect of exercise therapy (treadmill running) on *Rnf34* levels in dorsal horn (laminae I–II). Levels of *Rnf34* mRNA were determined by RT-PCR. Time course measurements were performed at 1, 3, and 6 weeks after surgery. The results were normalized against the corresponding levels of *Gapdh* mRNA, a housekeeping gene. Values represent means ± S.D. (n=6). *p < 0.05 according to Student's t-test and two-way ANOVA, followed by post-hoc Tukey HSD test. The main effect of time dependency yielded an F value of $F_{(2, 30)} = 7.3757$, $p = 0.00133$.

To confirm the laterality of *Rnf34* expression level, we separately analyzed the ipsilateral and contralateral sides of laminae I–II, as shown in Fig. 6. At 3 weeks after surgery, expression of *Rnf34* was lower on the ipsilateral side than on the contralateral side. A similar tendency was also detectable at 1 and 6 weeks after surgery, but the differences were not significant.
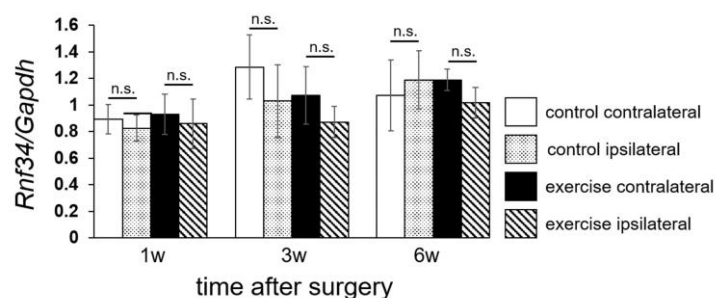
Figure 6. Laterality of *Rnf34* mRNA level in dorsal horn (laminae I–II). Levels of *Rnf34* mRNA, presented in Figure 5, were analyzed separately in the ipsilateral and contralateral sides. Values represent means ± S.D. (n = 6). No significant differences were detected by three-way ANOVA. The main effect of laterality yielded an F value of $F_{(1, 60)} = 2.471$, $p = 0.121$.

We also evaluated expression of *Pacap*. In the exercise group, expression of *Pacap* was significantly upregulated 1 week after surgery, but significantly downregulated 3 weeks after surgery, relative to the control group (Fig. 7). Expression of *Pacap* also tended to be downregulated 6 weeks after surgery, but the difference was not significant in comparison to the control group.
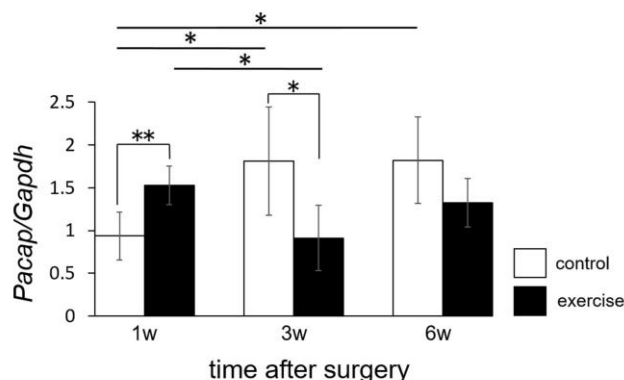
Figure 7. Effect of exercise therapy (treadmill running) on *Pacap* levels in dorsal horn (laminae I–II). Levels of *Pacap* mRNA were determined by RT-PCR. Time course measurements were performed at 1, 3, and 6 weeks after surgery. The results were normalized against the corresponding levels of *Gapdh* mRNA, a housekeeping gene. Values represent means ± S.D. (n = 6). *p < 0.05, **p < 0.01 according to Student's t-test and two-way ANOVA, followed by post-hoc Tukey HSD test. The interaction effect between exercise and time yielded an F value of $F_{(2, 30)} = 8.8204$, $p = 0.001$.

There was no significant difference in *Pacap* expression between the ipsilateral and contralateral sides (Fig. 8).
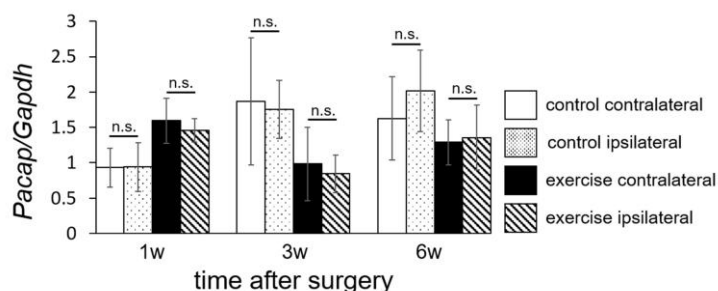
Figure 8. Laterality of *Pacap* mRNA level in dorsal horn (laminae I–II). Levels of *Pacap* mRNA, presented in Figure 5, were analyzed separately in the ipsilateral and contralateral sides. Values

198   represent means ± S.D. (n = 6). No significant differences were detected by three-way ANOVA. The
199   main effect of laterality yielded an F value of $F_{(1, 60)} = 1.0424$, $p = 0.3114$.

3. Discussion

201   In this study, we analyzed molecular changes in the spinal cord dorsal horn in order to clarify
202   the analgesic mechanism of exercise in the treatment of neuropathic pain. We employed the SNL
203   model in this study. This model has been demonstrated to produce neuropathic pain without motor
204   function loss [16]. Consistent with this, all of our SNL animals had maximal BBB score [17],
205   indicating no motor deficiency in the hind limbs.
206   We identified two genes, *Rnf34* and *Pacap*, whose expression levels in the dorsal horn were
207   changed by exercise. Ring finger protein 34 (RNF34) is a specific E3 ubiquitin ligase for PGC-1α and
208   one of human ortholog gene family. The protein was initially identified RING finger homologous to
209   IAP type (hRFI) [18, 19]. It was recently shown that the ring finger domain has E3 ubiquitin activity
210   that targets caspase-8 and -10 in death receptor–mediated apoptosis [19], and that exogenous
211   overexpression of hRFI in colorectal cancer cells inhibits the extrinsic apoptotic pathway [20].
212   Ubiquitin (Ub) ligation is implicated in active protein metabolism and subcellular trafficking, and its
213   impairment is involved in various neurologic diseases [21]. Jin et al. reported that RNF34 reduces the
214   expression of the γ2 GABA$_A$R subunit by increasing the ratio of ubiquitinated to nonubiquitinated
215   γ2. Overexpression of RNF34 in hippocampal neurons decreases the density of γ2 GABA$_A$R clusters
216   and the number of GABAergic contacts received by these neurons. shRNA-mediated knockdown of
217   endogenous *Rnf34* leads to elevated γ2 GABA$_A$R cluster density and GABAergic innervation. Jin et
218   al. concluded that RNF34 regulates postsynaptic γ2-GABA$_A$R clustering and GABAergic synaptic
219   innervation [22]. Although there has not been reported that inhibition of Rnf34 directly ameliorated
220   pain sensation, activation of GABA$_A$ receptor in spinal dorsal horn has been established to be one of
221   the most powerful analgesic mechanism in mammals. Pharmacologic removal of GABA$_A$
222   receptor-mediated neurotransmission elicited pronounced pain hypersensitivity in intact animals
223   [23]. GABA$_A$ receptor agonists have, therefore, been proposed as potent analgesics for pathological
224   pain [24]. Afrazi et al. reported that application of allopregnanolone, a neurosteroid, markedly
225   ameliorated diabetes-induced thermal hyperalgesia in rats via preservation of γ2 subunit of GABA$_A$
226   receptor in lumbar dorsal horn [25]. The working mechanism of this substance was inhibition of
227   GABA$_A$ receptor down-regulation. Preservation of GABA$_A$ receptors may shift the stimulation-pain
228   response from hypersensitive to hyposensitive in the patients with neuropathic pain. Therefore,
229   inhibition of *Rnf34* induces pain relief via preservation of GABA$_A$ receptors. As an alternative
230   hypothesis of the possible mechanism for exercise-induced analgesia, Rnf34 is mainly expressed in
231   oligodendrocytes in the CNS [26]. Therefore, it is possible that exercise improves
232   oligodendrocyte/axonal function [27]. Specific oligodendrocyte injury was recently shown to induce
233   neuropathic pain [28].
234   In this study, we demonstrated that expression of *Rnf34* in the dorsal horn, an area containing
235   postsynaptic receptor for GABAergic transmission, was inhibited by exercise 3 weeks after the
236   operation (Fig. 5). Because the GABAergic system is one of the most powerful endogenous analgesic
237   systems, suppression of RNF34 expression might provide pain relief via inhibition of postsynaptic
238   γ2-GABA$_A$R clustering under neuropathic pain conditions. We detected an exercise-induced
239   inhibition of *Rnf34* expression 3 weeks after the operation, in comparison with control animals,
240   although we observed no difference between the two groups at 1 or 6 weeks after the operation. This
241   may be because 1 week of exercise may be too brief to allow expression of analgesic reactions. On the
242   other hands, despite of persisted pain relief by exercise was continued until 6 weeks after the
243   operation (Fig. 3), the *Rnf34* mRNA level had risen nearly to the level in the control group at 6 weeks
244   (Fig. 5). Inhibition of *Rnf34* expression around 3 weeks after the operation may have decreased the
245   total amount of RNF34 protein in the dorsal horn, and this lower level of RNF34 protein may have
246   persisted until the end of observation (6 weeks after the operation).

247   PACAP（pituitary adenylate cyclase-activating polypeptide), a neuropeptide that stimulates
248 adenylate cyclase in rat anterior pituitary cell cultures, was originally isolated from ovine
249 hypothalamic tissues by Miyata et al. [29]. PACAP27 and PACAP38 are members of the
250 VIP/secretin/glucagon family of peptides that have diverse neuro-regulatory effects in
251 sympathoadrenal cell development and function [30]. In human cadavers, PACAP-like
252 immunoreactivity is detectable both in dorsal horn and dorsal root ganglia [31]. Narita et al.
253 reported that PACAP induces hyperalgesia in the mouse spinal cord, and detected PACAP38
254 immunoreactivity in numerous nerve fibers in the superficial layers of the dorsal horn of the cervical,
255 thoracic, lumbar, and sacral segments. Moreover, intrathecal application of PACAP38 elicits
256 pain-like behavior in mouse [32].

257   Zhang et al. observed *Pacap* mRNA expression in L5 dorsal root ganglion after unilateral
258 adjuvant-induced inflammation in the rat paw [33]. Mabuchi et al. reported that mice lacking the
259 *Pacap* gene (*Pacap*$^{-/-}$) do not exhibit inflammatory pain induced by intra-plantar injection of
260 carrageenan or neuropathic pain induced by L5 spinal nerve transection, although they do retain
261 normal nociceptive responses. Intrathecal administration of NMDA results in mechanical allodynia
262 in wild-type mice, but not in *Pacap*$^{-/-}$ mice [34]. Davis-Taber et al. reported that intrathecal
263 application of PACAP receptor antagonist potently reduces mechanical allodynia in a neuropathic
264 spinal nerve ligation model [35]. These reports indicate that inhibition of *Pacap* expression in dorsal
265 horn may relieve pain in patients or animals with neuropathic pain.

266   In this study, we demonstrated that the *Pacap* mRNA level in the exercise group was just
267 initially higher than that in the control group at 1 week (Fig. 7). In our research protocol, forced
268 exercise started the day after the operation. The animals in the exercise group may have experienced
269 higher stress due to the early initiation of exercise after surgery, causing a protein related to pain
270 mechanisms (PACAP) to be activated. On the other hand, in the exercise group, the *Pacap* mRNA
271 level decreased dramatically between 1 week and 3 weeks after the operation and *Pacap* mRNA
272 levels 3 and 6 weeks after the operation were lower in the exercise group than in the control group.
273 Thus, it may take a rather long time (3 weeks) to achieve pain relief via exercise treatment.
274 Suppression of *Rnf34* (Fig. 6) and *Pacap* (Fig 8) were observed not only on the injured (ipsilateral)
275 side, but also on the non-injured (contralateral) side 3 weeks after the operation. Thus, the molecular
276 change we observed in the dorsal horn was not target-specific. It is possible that the initial molecular
277 changes induced by exercise were generated at a more proximal level, e.g., in brain cortex or
278 hypothalamus, and that the analgesic signals subsequently spread in a peripheral direction with no
279 distinction between the injured and non-injured sides. The limitation of this study is that we
280 observed the molecular change only in the spinal cord. The molecular change in the patients/animals
281 with neuropathic pain should be occur not only in the spinal cord, but also other central nervous
282 tissue such as brain cortex, hypothalamus and hippocampus. The molecular change by exercise
283 should also be occur in other nervous tissue than spinal cord. Further experiment was necessary to
284 clear up the effect of *Rnf34* or *Pacap* as working mechanism of exercise therapy. To consider possible
285 correlation with other genes detected on the RNA-seq, further bioinformatics analysis, e.g. pathway
286 analysis and hierarchical clustering analysis should be performed in the current data set or in
287 various experimental conditions. Otherwise, it would be important to explore the changes in target
288 molecules such as GABA$_A$ receptor and PACAP receptor to reveal the analgesic mechanism for
289 clinical implementation in the future.

290   In summary, the current study aimed to investigate the working mechanism of endogenous
291 mediators during exercise in the pathophysiology of neuropathic pain. The results of this study
292 provides important clinical significances. The rehabilitation including muscle exercise is usually
293 hard for the patients with pain. Consequently, clarification of the pain relief mechanisms is valuable
294 to provide convincing and satisfactory explanation to the patients. Our results suggest that
295 pharmacological inhibition of *Rnf34* and *Pacap* can be candidates for the treatment of neuropathic
296 pain. The combination of exercise and pharmacological inhibition was also considered as more
297 potent pain treatment. Our study is potentially transferable to future human studies.

298 **4. Materials and Methods**

299 *4.1. Animals*

300   All experimental procedures were conducted in accordance with a protocol approved by the
301 Ethical Committee for Animal Experiments of Ehime University (#05NU73-2). A total of 72 female
302 Wistar rats (Charles River Laboratories, Yokohama, Japan) were purchased at 6 to 8 weeks old, and
303 randomly divided into three groups. L5 spinal nerve injury animals divided to two groups: with (the
304 exercise group) and without (the control group) exercise. In some animals, sham operation was
305 performed (the sham group). All rats were subjected to behavioral tests after surgery. The exercise
306 group underwent treadmill running. All rats were sacrificed to harvest spinal cord tissues for further
307 analyses, as described below.

308 *4.2 Surgical Procedures*

309   Rats were anesthetized with 1.5–2% (v/v) isoflurane in air, and then right L5 spinal nerve
310 ligation was performed as described by Kim and Chung [16]. After shaving of hair and sterilization
311 with iodine/70%ethanol, a midline longitudinal incision was made from the L4 to S1 vertebrae, and
312 the right paraspinal muscle was exposed. The paraspinal muscle was then removed from the level of
313 the L5 spinous process to the sacrum. The transverse process of L6 was exposed, and removed. The
314 L5 spinal nerve was tightly ligated with a piece of 5-0 silk distal to the L5 dorsal root ganglion. After
315 nerve ligation, the wound layer of the dorso-lumbar fascia and skin incision were closed with 5-0
316 silk thread. Sham operation was performed in the same manner, except that nerve ligation after
317 exposure was omitted.

318 *4.3 Evaluation of motor function*

319   Motor function was assessed with the Basso, Beattie, and Bresnahan (BBB) scoring scale [17],
320 one of the most widely used methods for evaluating hind-limb motor function in rats and mice, a
321 21-point scale that ranks no locomotion as 0 points and normal gait as 21 points. BBB scoring was
322 performed by three individuals who were unaware of the treatments that the rats had received. Data
323 reflect the averages of the three observers' scores.

324 *4.4 Evaluation of pain-like behavior*

325   To evaluate mechanical sensitivity of the foot, as determined by foot withdrawal threshold in
326 response to mechanical stimuli, we performed the von Frey test using Semmes Weinstein
327 Monofilaments (A835-14-18, SAKAI Medical, Tokyo, Japan). The rats were placed on a metal mesh
328 floor, and von Frey filaments were applied from underneath the metal mesh floor to the foot. To
329 determine the withdrawal threshold, the stimulus strength was sequentially increased and
330 decreased by up-down method. When the rats felt pain and withdrew their paw, the withdrawal
331 threshold was measured by applying forces 5.5 , 8.65, 11.7, 15 and 29 g. Paw sensitivity threshold
332 was defined as the minimum pressure at which immediate withdrawal reflex of the paw was
333 observed more than three times in a row. The measurements were performed before surgery and
334 weekly for 6 weeks after surgery (1w, 2w, 3w, 4w, 5w, and 6w). Thermal sensibility was assessed by
335 using the Hargreaves' plantar test apparatus (Ugo Basile, Varese, Italy) as previously described   [36,
336 37]. In brief, rats were placed on a 2mm thick glass floor 30 minutes before the experiment for
337 habituation. A heat generator with an aperture of 10mm diameter was focused onto the hindpaw
338 plantar surface pointing at both the lateral and the medial paw test sites. Thermal withdrawal
339 latency was taken as mean of three measurements per each hindpaw, with 5 min interval between
340 each measurement. The withdrawal latencies were recorded in seconds of each paw.

341 *4.5 Treadmill running*

342   The exercise group was assigned to perform interval training programs on a treadmill (MK-680,
343   Muromachi Kikai, Tokyo, JAPAN). Initial treadmill conditions were as follows: 10 m/min at 10
344   degrees inclination for 10 min. Treadmill running started the day after the operation, and was
345   performed 5 days per week. The velocity of the treadmill increased by 1 m/min each day until it
346   reached 20 m/min. During the experimental period, the exercised rats exhibited no significant
347   change of body weight in comparison with sedentary controls.

348   *4.6 Laser Micro-Dissection (LMD) for RNA extraction*

349   Spinal cords from the exercise, control, and sham groups were dissected without fixation,
350   immediately embedded in O.C.T. Compound (Tissue-Tek®, Sakura Finetek Japan, Tokyo, Japan),
351   and frozen in dry ice/acetone baths. Frozen sections 20 μm thick were processed, and portions of
352   laminae I–II in dorsal horn were clipped out using a laser microdissection system (Leica LMD7000,
353   Leica Microsystems, Tokyo, Japan). About 10 clipped slices of sections were collected per rat, and
354   these clipped sections were collected separately from the ipsilateral and contralateral sides. The
355   clipped sections were immediately subjected to RNA purification using an RNA isolation kit
356   (NucleoSpin® RNA XS, Clontech, TaKaRa, Shiga, Japan). The flow of the research protocol from
357   operation to mRNA extraction is shown in Figure 3.

358   *4.7 RNA Sequence and Functional Annotation Bioinformatics*

359   We selected six samples derived from the spinal cords of three rats each from the exercise and
360   control groups 6 weeks after surgery. The clipped sections obtained from both ipsilateral and
361   contralateral side were pooled. Isolated RNA (5 ng) was subjected to NGS library preparation using
362   the SMARTer® Stranded Total RNA Sample Prep Kit–Pico Input Mammalian (Clontech, TaKaRa).
363   Each library (16 pM) was subjected to 2 × 75-bp paired-end sequencing sequenced on an Illumina
364   MiSeq system using the MiSeq Reagent Kit v3–150 cycle (Illumina, San Diego, CA, USA).
365   Bioinformatics analysis was performed using the following software: Tophat for gene mapping of
366   the sequence data, Cufflinks to determine differences in expression levels, and DAVID
367   Bioinformatics Resources 6.8 (National Institute of Allergy and Infectious Diseases [NIAID], NIH,
368   Bethesda, MD, USA) for functional annotation of the gene list for coding mRNAs.

369   *4.8 RT-PCR*

370   Extracted total RNA was subjected to first-strand cDNA synthesis with random primers using
371   the SuperScript®VILO cDNA Synthesis Kit (Thermo Fisher, Waltham, MA, USA). Products of
372   reverse transcription were diluted 20-fold and used as templates for quantitative real-time PCR
373   analysis (qRT-PCR) using SYBER Premix Ex Taq TMII (Tli RNase H Plus) on a 7500 Real Time PCR
374   System (Applied Biosystems, Foster City, CA, USA), using cDNA derived from the exercise and
375   control groups at 1, 3, and 6 weeks after surgery. Detected signals were confirmed as specific by a
376   dissociation protocol. Data were normalized against the corresponding expression levels of *Gapdh*.
377   Primer sets used for qRT-PCR were as follows:
378   *Rnf34* forward: CAGTCTGCTATGGTGCTGAGTT,
379   *Rnf34* reverse: TAGAGGTAGCACCCGCCTTCAT,
380   *Pacap* forward: CCTACCGCAAAGTCTTGGAC,
381   *Pacap* reverse: TTGACAGCCATTTGTTTTCG,
382   *Gapdh* forward: GAACATCATCCCTGAATCCA,
383   *Gapdh* reverse: CCAGTGAGCTTCCCGTTC

384   *4.9 Statistical Analysis*

385   Wilcoxon signed-rank test was used to analyze pain reactions (pain thresholds) by mechanical
386   stimulation. Student's t-test was used for two-sample analyses to determine whether mRNA

expression levels differed significantly between the exercise and control groups. Multi-way ANOVA (Analysis of Variance) was used for all of the RT-PCR data. Post-hoc Tukey HSD (Honestly Significant Difference) test was used to analyze the differences among the changes with time.

5. Conclusion

We demonstrated that pain relief in the SNL rats was achieved by 3 weeks of forced exercise, and that mRNA levels of Rnf34 and Pacap in the dorsal horn decreased relative to those in the no-exercise group. In this study, we employed next-generation sequencing in combination with laser microdissection methods in order to reveal the possible mechanism of exercise therapy in neuropathic pain, followed by the time course analysis of qPCR. To the best of our knowledge, such a comprehensive analysis of gene expression in dorsal horn where nociceptive information is processed is firstly reported here. We conclude that *Rnf34* and *Pacap* have been identified as potential candidates to imply direct and/or indirect correlation with pain-like behavior.

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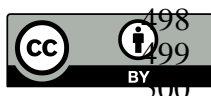
Conflicts of Interest: The authors declare no conflicts of interest.

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