Differential expression of rat hippocampal microRNAs in two rat models of chronic pain

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Abstract. The two most common forms of chronic pain are inflammatory pain and neuropathic pain. Nevertheless, the underlying mechanisms of these pain conditions and their therapeutic responses are poorly understood. MicroRNAs (miRNAs) negatively regulate cell genes, and thus control cell proliferation, inflammation and metabolism. In the present study, we examined gene expression in the hippocampus of rats in two models of chronic pain. In addition, we used the left hindpaw procedure to identify differences in the bilateral hippocampus. We divided the rats into the 4 following groups: the group with chronic constriction injury (CCI), the sham-operated group, the group injected with complete Freund's adjuvant (CFA) and the group injected with normal saline. miRNA expression profiles were analyzed using TaqMan low-density array (TLDA). We observed 54 miRNAs (22.7%) in the rats with CCI rats that were differentially expressed, including 7 miRNAs that were downregulated compared with the sham-operated rats. In the CFA-injected rats, 40 miRNAs (16.8%) were differentially expressed, including 8 miRNAs that were downregulated compared with the normal saline-injected rats. Pearson's correlation co-efficient for all detected miRNAs in the rat hippocampus failed to identify differences between the hippocampi bilaterally. An unsupervised cluster analysis produced separate clusters between the control and experimental groups. In this study, we demonstrate the differential expression of hippocampal miRNAs in two rat models of chronic pain; however, no significant differences were observed bilaterally in hippocampal miRNA expression. Further research is required to determine the correlation among miRNAs, messenger RNAs (mRNAs) and proteins.

Introduction

Chronic pain that is severe and difficult to manage can degrade the quality of life of patients. The two most common forms of chronic pain are inflammatory pain and neuropathic pain. These two conditions show similar clinical symptoms; however, their underlying mechanisms and responses are dissimilar. In general, chronic pain involves changes in the expression of messenger RNAs (mRNAs) or proteins that regulate cytokines, receptors and neurotransmitters in the dorsal root ganglia and the brain (1,2). Previous studies have suggested that the differential expression of multiple pain-associated genes plays a key role in the development and maintenance of chronic pain (3-5).

The hippocampus, a brain region that participates in learning and memory formation, plays a fundamental role in pain perception (6,7). The association between the hippocampus and pain has been the focus of a number of recent studies. In particular, recent studies have indicated that peripheral neuropathy alters hippocampal gene expression, which suggests that the hippocampus may contribute to neuropathic pain symptoms (8-10).

There are a few studies, however, that directly examine the association between the hippocampus and inflammatory or neuropathic pain. Furthermore, few studies have examined the role that hippocampal microRNAs (miRNAs) may play in chronic pain, particularly for the two different pain models. We hypothesized that hippocampal miRNA expression may differ between inflammatory and neuropathic pain models. An improved understanding of the underlying mechanisms for chronic pain may lead to more effective treatment strategies.

In this study, we used TaqMan® low-density array (TLDA) and quantitative real-time PCR (qRT-PCR) of miRNAs to examine miRNA expression in the hippocampus in two rat models of chronic pain. The model of neuropathic pain comprised rats with chronic constriction injury (CCI), whereas the model of inflammatory pain comprised rats injected with complete Freund's adjuvant (CFA), as previously described (11,12). In addition, we examined differences in gene expression between the hippocampus bilaterally, as bilateral effects have been observed with unilateral chronic pain models induced by inflammation or by ischemic monomelic neuropathy (13).

Materials and methods

All experimental procedures were approved by the Institutional Committee on Laboratory Animals of Nippon Medical School, Tokyo, Japan (approval no. 19-091) and were performed under the guidelines of the International Association for the Study of Pain.

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Male Sprague-Dawley rats (6-7 weeks of age and 200-250 g in weight; Experimental Animal Center, Saitama, Japan) were used. The rats were housed in clear plastic cages with sawdust bedding at standard room temperature under a 12-h light/dark cycle. All rats received food and water *ad libitum*. We divided the rats into 4 experimental groups as follows: i) a group of rats with CCI that were sacrificed 7 days after the procedure (CCI group, n=6); ii) a group of sham-CCI operated rats (sham-CCI group, n=6) [as previously described (11)]; iii) a group of rats injected with CFA that were sacrificed 7 days after the procedure (CFA group, n=6); and iv) a group of normal saline-injected rats (sham-CFA group, n=6) [as previously described (12)]. All surgical procedures were performed under deep anesthesia with sodium pentobarbital [50 mg/kg intraperitoneal (i.p.) injection].

Rat model of neuropathic pain induced by CCI. The CCI model was established according to the methods of Bennett and Xie (11). The left (ipsilateral) common sciatic nerve was exposed and loosely ligated with 4-0 silk thread at 4 regions that were spaced at approximately 1-mm intervals. The sciatic nerve in the sham-operated group was similarly exposed but not ligated.

CFA injection for the induction of inflammatory pain. The CFA model was established by injecting CFA (50% in saline, 20 μl) into the plantar surface of the left hindpaw. In the sham-CFA group, normal saline was similarly injected into the left hindpaw as previously described (12).

Behavioral assessment. The Plantar Test apparatus (Ugo Basile, Comerio, Italy) was used to examine thermal hyperalgesia, and the von Frey test (Muromachi Kikai, Tokyo, Japan) was used to examine mechanical allodynia. These tests were performed on the day before surgery (day 0), and on post-operative days 1, 3, 5, 7, 9, 11, 13 and 15, as previously described (14). For the plantar test, each rat was placed on a glass plate with radiant heat equipment (a 50-W halogen reflector bulb) underneath. After the acclimation period, radiation heat was applied to either the contralateral or ipsilateral hindpaw pad. The latency of paw withdrawal from the thermal stimulus was measured 3 times at 5-min intervals, and the average value of these 3 measurements was used as the response latency. For the von Frey test, each rat was placed on a metallic mesh floor, covered with a plastic box, and a von Frey filament with bending forces from 2.0 to 32.0 g was applied from under the mesh floor to the plantar surface of either the contralateral or ipsilateral hindpaw. In individual trials, each paw was stimulated with each filament 5 times at 10-sec intervals. The weakest force (g) that induced the withdrawal of the stimulated paw at least 3 times in each trial was considered the paw withdrawal threshold.

miRNA profiling. On post-operative day 7, the rats were deeply anesthetized with pentobarbital (300 mg/100 g body weight, i.p.) and decapitated immediately. The brains were rapidly removed, and the hippocampi were dissected as previously described (15). The unilateral hippocampi were divided into left and right sections. Each sample was placed in RNA later® (Applied Biosystems, LLC, Foster City, CA, USA) and stored at -80˚C until use. Total RNA was isolated using a mirVana™ miRNA isolation kit® (Applied Biosystems, LLC) according to the manufacturer's instructions. RNA quantity and quality were assessed using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). All RNA samples with an A260/280 nm reading of 1.8 were used for quantitative analysis. Total RNA samples containing miRNAs were used for qRT-PCR.

The miRNA expression profiles were analyzed using TLDA Rodent MicroRNA cards v.3 A and B (Applied Biosystems, LLC). Each card contains 373 pre-loaded rodent miRNA targets, which are all catalogued in the miRBase database (16) and 3 endogenous controls. The procedures were performed as previously described (17,18). Briefly, TLDAs were performed using a two-step process. First, 800 ng total RNA per sample was reverse transcribed using Megaplex RT primer pool A and B, which contains up to 381 stem-looped primers per pool, and a TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, LLC). Second, the resulting complementary DNA was diluted, mixed with TaqMan Universal PCR master mix (Applied Biosystems, LLC), and deionized in distilled water (Wako, Tokyo, Japan) and loaded into 1 of the 8 fill ports on the TLDA microfluidic cards. The cards were briefly centrifuged for 1 min at 1,600 x g to distribute samples to the multiple wells connected to the fill ports, and then sealed to prevent well-to-well contamination. Finally, the cards were processed and analyzed using a 7900 HT Real-Time PCR System (Applied Biosystems, LLC).

Data analysis was performed using DataAssist software v2.0 (Applied Biosystems, LLC). The data were represented as the threshold cycle (Ct) values, where Ct represents a unitless value defined as the fractional cycle number at which the sample fluorescence signal passes a fixed threshold above baseline. For each miRNA, the expression level was calculated using the comparative Ct method ($\Delta\Delta$Ct) and was further analyzed by comparing the fold change to the basal levels in the control samples. ΔCt was the difference in the Ct values derived from the experimental samples and the control, and $\Delta\Delta$Ct represented the difference between paired samples, as calculated by the following formula: $\Delta\Delta Ct = \Delta Ct$ of sample after surgical procedure $-\Delta Ct$ of the control. The expression ratio shows the relative quantity of the target gene (Xtarget) to the control gene (Xcontrol). The fold change was computed by the formula $Xtarget/Xcontrol = 2^{-\Delta\Delta Ct}$. For miRNAs, graphic displays were visualized as heat map results of hierarchical clustering. Distances between the samples and assays were calculated for hierarchical clustering based on ΔCt values using the Euclidean distance.

Statistical analyses. Values are expressed as the means ± standard deviation. A two-tailed paired t-test was used to compare latencies or threshold values in behavioral tests between the ipsilateral and contralateral sides (CCI group; n=6). Dunnett's test for multiple comparisons was used to compare latencies and threshold values obtained in the behavioral tests performed pre-operatively (day 0, n=6) with those obtained post-operatively (days 1, 3, 5, 7, 9, 11, 13 and 15; n=6). ANOVA followed by Tukey's test were performed using KyPlot 5.0 software (KyensLab Inc., Tokyo, Japan). Using Pearson's correlation, we assessed whether the hippocampal miRNAs that showed expression changes in the rat models of chronic pain were differentially expressed between the left and right hippocampus.

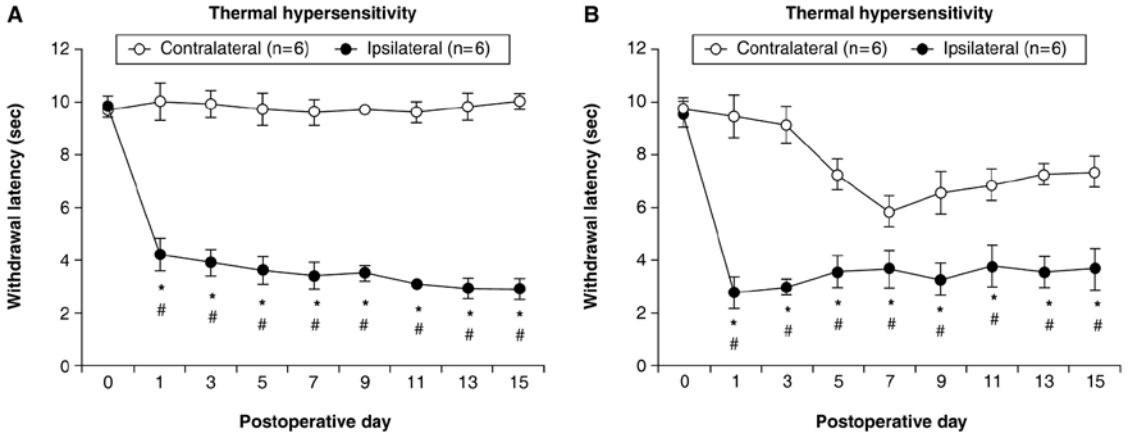

Figure 1. Effects of (A) chronic constriction injury (CCI) (n=6) and (B) complete Freund's adjuvant (CFA) injection (n=6) on thermal hypersensitivity. Thresholds for foot withdrawal on the ipsilateral and contralateral sides in response to thermal stimuli applied to the corresponding hindpaw pad in rats with CCI and CFA-injected rats. *P<0.01 compared with values on the contralateral side on the same days by a paired t-test; #P<0.01 compared with the values on the pre-operative day (day 0) by Dunnett's multiple comparisons. Values are the means ± standard error of the mean.

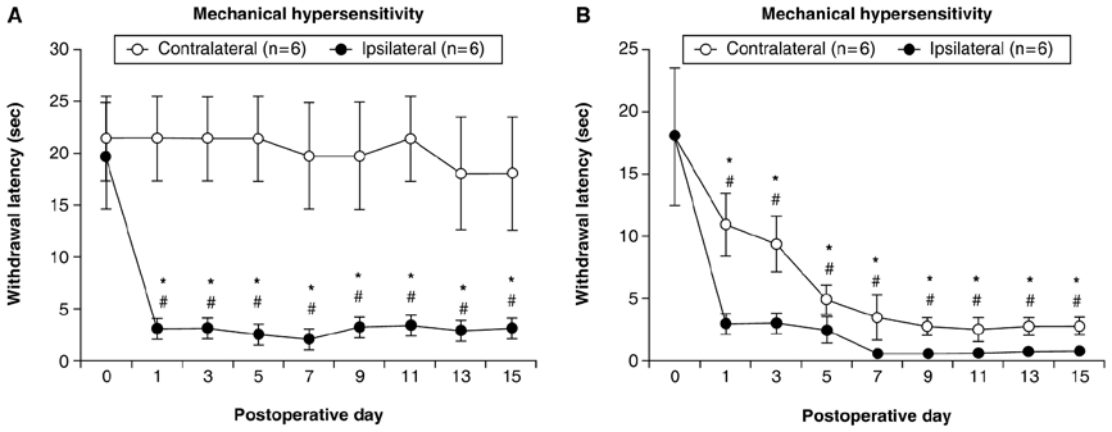

Figure 2. Effects of (A) chronic constriction injury (CCI) (n=6) and (B) complete Freund's adjuvant (CFA) injection on mechanical allodynia. Thresholds for foot withdrawal on the ipsilateral and contralateral sides in response to mechanical stimuli applied to the corresponding hindpaw pad in rats with CCI and CFA-injected rats. *P<0.01 compared with values on the contralateral side on the same days, by a paired t-test; #P<0.01 compared with the values on the pre-operative day (day 0) by Dunnett's multiple comparisons. Values are the means ± standard error of the mean.

Results

Behavioral assessment. The rats with CCI showed a significant decrease in the latency of paw withdrawal in the plantar test and threshold values in the von Frey test for the ipsilateral side on post-operative days (1, 3, 5, 7, 9, 11, 13 and 15) compared with day 0 (P<0.05). On the contralateral side, significant decreases were not observed (Figs. 1A and 2A).

Even though the CFA injection was administered to the left hindpaw, the rats with CFA showed a significant decrease in the latency of paw withdrawal in the plantar test, and a significant decrease in threshold values in the von Frey test for the hindpaws bilaterally. These differences were observed on the post-operative days examined compared with day 0 (P<0.05) (Figs. 1B and 2B).

TLDA. Of the 373 rat miRNAs, 237 (63.5%) were detected in the rat hippocampus. In the control rats, we observed no difference in miRNA expression between the left and right hippocampus (correlation value, 0.99; P<0.0001). Compared with the sham-operated rats, TLDA in the rats with CCI identified 54 miRNAs (22.7%) that were differentially expressed, including 7 miRNAs that were downregulated (Table I).

Compared with the normal saline-injected rats, the CFA-injected rats had 40 miRNAs (16.8%) that were differentially expressed, including 25 miRNAs that were downregulated (Table II).

Twenty miRNAs were expressed in both the rats with CCI and the CFA-injected rats, whereas 34 miRNAs were expressed in only the rats with CCI, and 22 were expressed in only the CFA-injected rats. There were no significant differences observed in the comparisons between the sham-operated rats and the normal saline-injected rats.

We observed no difference between the hippocampi bilaterally for either the CCI group (correlation value, 0.99; P<0.0001) or the CFA group (correlation value, 0.98; P<0.0001). Furthermore, there was no difference bilaterally among the control rats, the rats with CCI or the CFA-injected rats (Fig. 4). A clustergram of the samples and the miRNAs that showed significant differences are shown as a heat map in

Table I. The 54 miRNAs that were differentially expressed in the CCI group compared with the sham-operated group.

Assay	Fold change ± SD	P-value
hsa-miR-324-3p	1.64±0.40	<0.001
mmu-miR-125a-5p	2.10±0.24	<0.001
mmu-miR-132	2.11±0.46	<0.001
mmu-miR-151-3p	3.51±0.18	<0.001
mmu-miR-17	1.77±0.13	<0.001
mmu-miR-181c	0.51±0.21	<0.001
mmu-miR-191	3.06±1.20	<0.001
mmu-miR-222	2.38±0.44	<0.001
mmu-miR-29c	0.37±0.29	<0.001
mmu-miR-31	2.04±0.43	<0.001
mmu-miR-320	1.76±0.54	<0.001
mmu-miR-434-3p	3.35±0.78	<0.001
mmu-miR-539	2.24±0.68	<0.001
rno-miR-345-3p	2.48±0.37	<0.001
rno-miR-381	0.25±0.31	<0.001
hsa-miR-30a-3p	1.83±0.51	<0.01
hsa-miR-30e-3p	1.55±0.40	<0.01
mmu-miR-126-3p	1.96±0.28	<0.01
mmu-miR-133a	2.48±1.55	<0.01
mmu-miR-140	1.65±0.25	<0.01
mmu-miR-150	2.04±0.98	<0.01
mmu-miR-212	2.14±0.74	<0.01
mmu-miR-30a	1.42±0.18	<0.01
mmu-miR-323-3p	1.96±0.58	<0.01
mmu-miR-331-3p	1.42±0.14	<0.01
mmu-miR-383	2.02±1.07	<0.01
mmu-miR-431	1.63±0.46	<0.01
mmu-miR-487b	1.45±0.36	<0.01
mmu-miR-770-5p	1.82±0.64	<0.01
mmu-miR-872#	1.92±0.50	<0.01
rno-miR-125b#	2.09±0.61	<0.01
rno-miR-146B	1.74±0.41	<0.01
rno-miR-339-3p	3.21±1.57	<0.01
rno-miR-409-3P	1.75±0.51	<0.01
rno-miR-504	1.76±0.56	<0.01
rno-miR-632	0.58±0.23	<0.01
rno-miR-664	1.70±0.50	<0.01
rno-miR-7a#	1.85±0.63	<0.01
hsa-miR-28-3p	2.17±0.43	<0.05
hsa-miR-423-3P	1.65±0.41	<0.05
mmu-miR-128a	1.61±0.23	<0.05
mmu-miR-134	1.43±0.31	<0.05
mmu-miR-138	2.07±0.95	<0.05
mmu-miR-186	2.54±2.32	<0.05
mmu-miR-193b	1.98±1.10	<0.05
mmu-miR-204	1.81±0.84	<0.05
mmu-miR-23b	1.53±0.19	<0.05
mmu-miR-24	1.57±0.40	<0.05
mmu-miR-30d	0.63±0.16	<0.05
mmu-miR-325	0.58±0.44	<0.05
mmu-miR-376b	0.58±0.26	<0.05
mmu-miR-380-5p	1.45±0.41	<0.05
mmu-miR-384-5p	1.62±0.56	<0.05
rno-miR-351	2.24±0.54	<0.05

Values are the mean fold change ± SD in each group. CFA, complete Freund's adjuvant;; miRNA, microRNA; mmu, *Mus musculus*; rno, *Rattus norvegicus*; P-value, statistical results of Tukey's test.

Table II. The 40 miRNAs that were differentially expressed in the CFA group compared with the normal saline group.

Assay	Fold change ± SD	P-value
hsa-miR-324-3p	1.53±0.30	<0.001
mmu-miR-15b	1.09±0.26	<0.001
mmu-miR-191	3.06±0.90	<0.001
mmu-miR-326	0.52±0.50	<0.001
mmu-miR-770-5p	1.85±0.46	<0.001
rno-miR-409-3P	1.63±0.50	<0.001
hsa-miR-223	2.83±2.23	<0.01
hsa-miR-30a-3p	1.86±1.08	<0.01
mmu-miR-125a-5p	2.10±0.50	<0.01
mmu-miR-126-3p	1.96±0.35	<0.01
mmu-miR-133a	2.48±0.92	<0.01
mmu-miR-134	1.43±0.38	<0.01
mmu-miR-138#	1.98±0.80	<0.01
mmu-miR-139-5p	1.96±0.51	<0.01
mmu-miR-148b	1.03±0.41	<0.01
mmu-miR-212	2.06±1.20	<0.01
mmu-miR-296-5p	0.61±0.19	<0.01
mmu-miR-652	1.13±0.16	<0.01
mmu-miR-708	1.48±0.23	<0.01
mmu-miR-872	1.00±0.13	<0.01
rno-miR-146B	1.77±0.62	<0.01
rno-miR-219-2-3p	0.83±0.15	<0.01
rno-miR-664	1.45±0.55	<0.01
rno-miR-7a#	1.49±0.58	<0.01
hsa-miR-140-3p	1.54±0.45	<0.05
hsa-miR-30e-3p	1.37±0.44	<0.05
mmu-miR-142-3p	0.70±0.06	<0.05
mmu-miR-146a	2.27±0.88	<0.05
mmu-miR-150	2.04±0.68	<0.05
mmu-miR-219	0.87±0.23	<0.05
mmu-miR-27a	0.98±0.16	<0.05
mmu-miR-324-5p	0.90±0.08	<0.05
mmu-miR-375	0.78±0.37	<0.05
mmu-miR-383	2.02±0.57	<0.05
mmu-miR-539	2.24±0.64	<0.05
mmu-miR-7a	1.03±0.21	<0.05
mmu-miR-872#	2.40±1.48	<0.05
rno-miR-125b#	2.05±1.02	<0.05
rno-miR-204#	2.52±0.79	<0.05
rno-miR-504	1.30±0.18	<0.05

Values are the mean fold change ± SD in each group. CFA, complete Freund's adjuvant; miRNA, microRNA; mmu, *Mus musculus*; rno, *Rattus norvegicus*; P-value, statistical results of Tukey's test.

Fig. 3. As can be observed in the figure, the heat map indicates 3 main branches (control and experimental) that separate the CCI from the CFA group.

Discussion

In the present study, we determined that hippocampal miRNA expression differed in two pain models that have similar symptoms (CCI and CFA models). Furthermore, we demonstrated

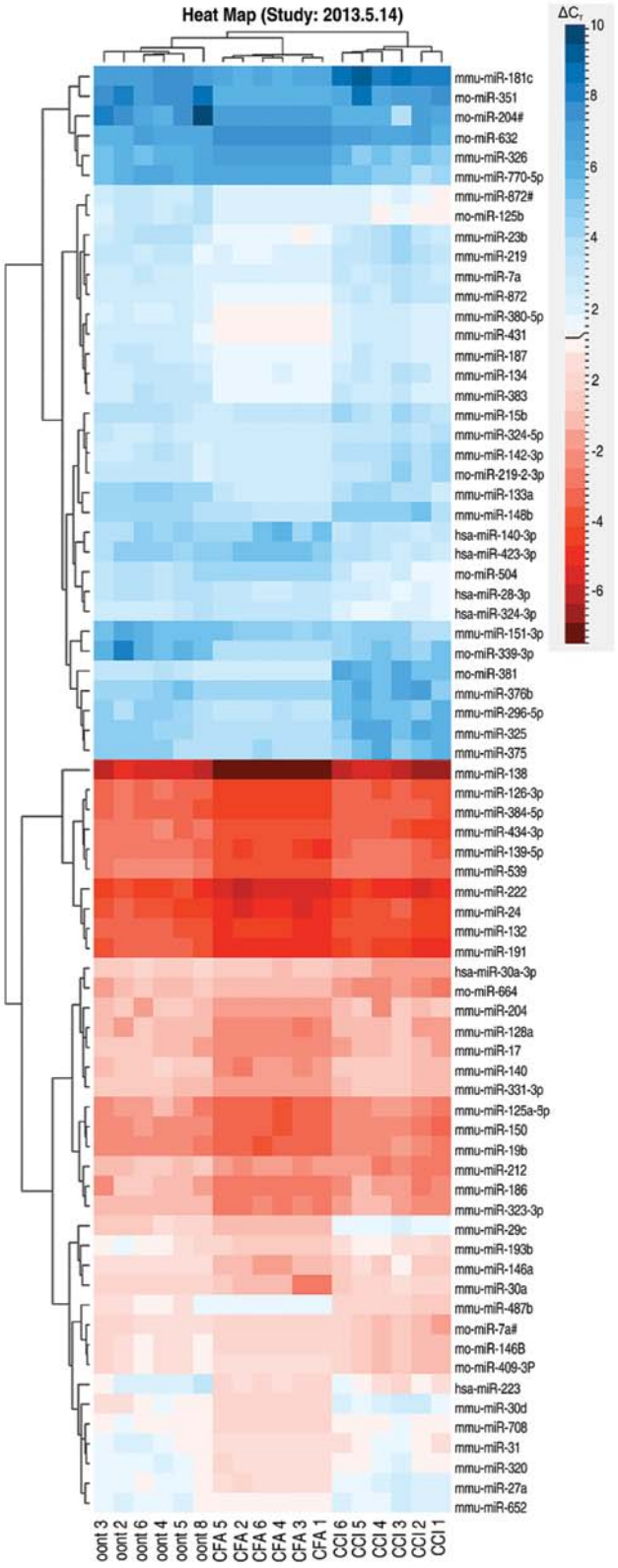

Figure 3. Unsupervised hierarchical cluster analysis using Euclidean distance from TaqMan low-density arrays. There were 73 microRNAs differentially expressed in the control and chronic pain models. Colors show the level of gene expression; red indicates a higher expression, blue indicates lower expression. Each group is shown separately: control group, chronic constriction injury (CCI) group and complete Freund's adjuvant (CFA) group.

that miRNA expression did not differ between the left and right hippocampus.

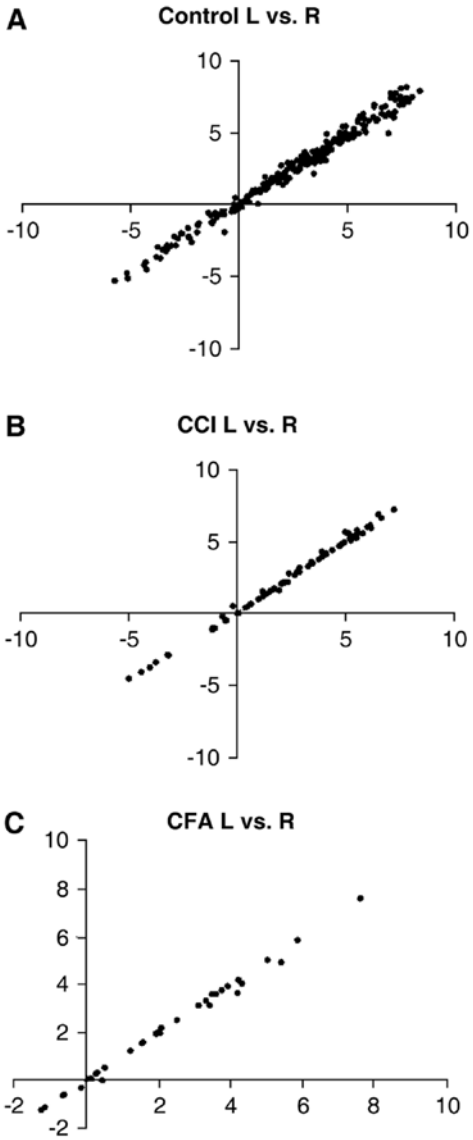

Figure 4. Pearson's correlation was used to assess hippocampal miRNA expression. Left hippocampal sample ΔCt (difference in threshold cycle) is plotted on the abscissa and the right sample ΔCt is plotted on the ordinate. In the control rats, there was no difference in miRNA expression between the left and right hippocampus (correlation value, 0.99; P<0.0001), in the chronic constriction injury (CCI) group (correlation value, 0.99; P<0.0001), and in the complete Freund's adjuvant (CFA) group (correlation value, 0.98; P<0.0001).

Pain hypersensitivity and dysesthesia. In both the CCI and CFA models, we observed hyperalgesia and mechanical allodynia on the injured side beginning on the first post-operative day (day 1). Pain hypersensitivity continued until day 14, which indicated that a similar chronic pain was induced in the two models. In the CFA model, we observed hyperalgesia and allodynia on the uninjured side beginning on day 3. We suspect that this was due to CFA-mediated inflammation that was caused by systemic inflammation, which led to pain hypersensitivity on the uninjured side.

miRNA expression. On day 7, when chronic pain was thought to be complete, we investigated hippocampal miRNA expression using cluster analysis.

Cluster analysis is a method used for grouping samples same with very similar expression levels; it aids in the identification of group-specific expression patterns. We observed differential miRNA expression in the hippocampus. Furthermore, the CCI and CFA group each produced differential miRNA expression patterns.

The limbic system, which includes the hippocampus, has been reported to be associated with memory and emotional responses (19). Both the cause and persistence of chronic pain is complex and cannot be easily explained. Thus, differences at the miRNA level are likely involved, even when the symptoms of chronic pain are identical. Therefore, treatments for the different forms of chronic pain may not be the same. We believe that our findings may lead to the future assessment of treatment methods, and the development of therapeutic agents with greater efficacies.

Difference between the left and right hippocampus. Previous studies have indicated a correlation between chronic pain and the hippocampus (20). Although previous studies have examined anatomical differences between the hippocampus bilaterally, as well as the effect of peripheral pain on the hippocampus, the responsible pathways remain unelucidated. Therefore, we in this study, investigated how unilateral chronic pain affects the hippocampus bilaterally. In addition, we examined the hypothesis that a model of inflammatory pain would induce a systemic reaction, but not bilateral differences in the hippocampus. In both the CCI and CFA groups, we searched for bilateral differences in miRNA expression that showed significant changes. We found no evidence that indicated a difference between the hippocampus bilaterally. We concluded that the bilateral hippocampal miRNA expression is equal in the two chronic pain models.

Although the hippocampus functions bilaterally, it is widely known that its asymmetrical nature is an essential feature for high-order brain functioning (21,22). Bilateral differences in hippocampal projections related to chronic pain have not been investigated thus far. Although bilateral differences in miRNA expression have been previously demonstrated in the spinal cord, in this study, to our knowledge, we show for the first time that there was no difference in miRNA expression between the left and right hippocampus.

Symptomatic bilateral differences disappeared in the model of inflammatory pain (CFA group), but remained in the CCI model. In both models, however, we observed no differences between the left and right hippocampus. In the pain pathway, the left and right sides of the hippocampus are thought to be morphologically connected or in a compartment, and thus, symptomatic bilateral differences were not affected.

In conclusion, two chronic pain models that show similar pain actions also showed differential changes in hippocampal miRNA expression patterns. Such a finding indicates that each model may possess a unique mechanism for regulating mRNA and protein expression. We observed no differences in miRNA expression between the left and right hippocampus. Thus, we believe that clarifying the mechanisms underlying pain development may lead to improved treatment techniques, as well as to the development of therapeutic agents with greater efficacies.

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