microRNA changes in the dorsal horn of the spinal cord of rats with chronic constriction injury: A TaqMan® Low Density Array study

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Abstract. Elucidation of the mechanisms underlying neuropathic pain is expected to aid in the discovery and selection of effective therapeutic methods. Currently, microRNA (miRNA) is thought to play an important role in the development and maintenance of the nervous system. We, therefore, hypothesized that miRNAs are involved in neuropathic pain, and investigated this possibility by analyzing miRNA expression in the dorsal horn of the spinal cord in a chronic constriction injury (CCI) rat model using the TaqMan® Low Density Array (TLDA). Neuropathic pain model rats were produced by CCI induced by ligation of the sciatic nerve. The miRNA expression in the dorsal horn of the spinal cord was analyzed in Day 0 rats, with no sciatic nerve ligation or sham operation, Day 7 rats, examined 7 days after sciatic nerve ligation or sham operation, and Day 14 rats, examined 14 days after sciatic nerve ligation or sham operation using TLDA. In this study, 111 miRNAs were significantly regulated in CCI rats in both the Day 7 and Day 14 groups compared with sham rats in both groups. Of these 111, there were 75 miRNAs (67.6%) that had been analyzed in previous reports and 36 miRNAs (32.4%) related to the development of tumors of the nervous system and neurodegenerative diseases. Certain miRNAs were reported to be related to neuropathic pain; miR-500, -221 and -21. The expression levels of a large number of miRNAs in the dorsal horn of the spinal cord in CCI rats changed. These results provide a step toward elucidation of the mechanisms underlying neuropathic pain.

Introduction

Neuropathic pain is a condition that is refractory to analgesics and significantly decreases the quality of life. Elucidation of the mechanisms underlying neuropathic pain represents the first step in the discovery and selection of effective therapies for this condition. In recent years, the presence of non-coding RNA, termed microRNA (miRNA), consisting of approximately 20 bases has become a major focus of research. It is now known that miRNA binds to the 3'-untranslated region of its target mRNA. Binding of miRNA to a target that has partially complementary sequences inhibits mRNA translation, and binding to a target that has perfectly complementary mRNA contributes to the degradation of the mRNA (1-4). It has been reported that a large number of these small RNAs exist in the mammalian nervous system and that they play an important role in nerve generation and degeneration (5,6).

The dorsal horn of the spinal cord is the location of the secondary neurons that receive nociceptive stimuli in neuropathic pain. The dorsal horn plays an important role in pain control and treatment as well as in the generation of neuropathic pain and is also responsible for central sensitization and inhibition of the transmission of nociceptive stimuli via the descending inhibitory system. It is therefore presumed that many miRNAs play a role in the dorsal horn of the spinal cord in neuropathic pain. Although it has been suggested that determination of global changes in miRNA would help to clarify the mechanisms underlying neuropathic pain, a comprehensive analysis of miRNA expression in the dorsal horn of the spinal cord in chronic constrictive injury (CCI) model rats has not been reported. Previous reports have demonstrated that some miRNA expression is associated with pain (7-10). Thus, we comprehensively analyzed miRNA expression in the dorsal horn of the CCI rat model using the TaqMan® Low Density Array (TLDA).

Materials and methods

Experimental animals. All experimental procedures were approved by the Institutional Committee on Laboratory Animals of Nippon Medical School (approval no. 22-162) and were performed under the guidelines of the International Association for the Study of Pain (11).

Male Sprague-Dawley rats (n=30; 6-7 weeks old; weight, 200-250 g; Saitama Experimental Animals, Saitama, Japan)

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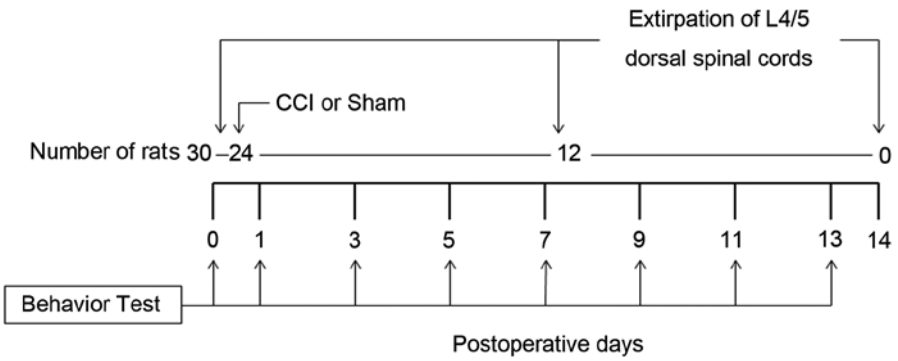

Figure 1. Experimental protocol. Neuropathic pain was produced by chronic constrictive injury of the sciatic nerve. Behavioral tests comprised the plantar test and a test using von Frey filaments. In the Day 0 group, the L4/5 dorsal spinal cord was removed after behavioral testing. In the Day 7 and Day 14 group CCI rats, the sciatic nerve was ligated after behavioral testing. In the Day 7 and Day 14 group sham-operated rats, a sham operation with no ligation of the sciatic nerve was performed after behavioral testing. All rats in the Day 7 and Day 14 groups underwent surgical removal of the L4/5 dorsal spinal cord at 7 and 14 days after sciatic nerve ligation or sham operation, respectively.

were used for all experiments. Experimental neuropathy was produced as described elsewhere (12-15). In the present study, a sciatic nerve injury model was used. All surgical procedures were performed on rats that were deeply anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally).

To assess pain thresholds, we performed the plantar test and the von Frey behavioral tests. The details of the test methods have been discussed in previous reports (12,14-16). Briefly, the plantar test (Ugo Basile, Comerio, Italy) was used to examine thermal hyperalgesia. Mechanical allodynia was measured using a set of von Frey filaments (Muromachi Kikai, Saitama, Japan) with bending forces ranging from 0.04 to 72.0 g.

Following behavioral testing, the 30 rats were divided into three groups. In the Day 0 group rats (n=6), that did not undergo any operation, the L4/5 spinal cord was removed immediately following behavioral testing. The Day 7 group rats (n=12) underwent either sciatic nerve ligation (CCI model rats, n=6) or sham operation (n=6). Seven days after the operation and the behavioral testing, the L4/5 spinal cord was removed. Day 14 group rats (n=12) underwent either sciatic nerve ligation (n=6) or sham operation (n=6). Fourteen days after the operation and the behavioral testing, the L4/5 spinal cord was removed. The behavioral test was practiced at some point after the operation (nerve ligation or sham). The experimental protocol is shown in Fig. 1. A two-tailed t-test was used to compare the latencies or threshold values in behavioral tests between CCI and sham rats. A one-way analysis of variance (ANOVA) followed by Tukey's test was used to compare latencies or threshold values obtained in behavioral tests performed on Day 0 (undergoing no operation; n=6) and at some point after the operation.

Extirpation and preservation of samples. The rats were deeply anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and the L4/5 spinal cords were dissected. The L4/5 spinal cords were washed twice with cold phosphate-buffered saline (PBS) and immediately divided into contralateral (non-ligated side or right side) and ipsilateral (ligated side or left side) samples, and stored in RNAlater solution (Applied Biosystems, Foster City, CA, USA). Each ipsilateral sample was further divided into ventral and dorsal samples and the dorsal spinal cords were stored at -20˚C in RNAlater solution.

Total RNA isolation/miRNA isolation. The samples were defrosted and RNAlater solution was rapidly separated from the samples. Total RNA was extracted using a mirVana miRNA Isolation kit (Applied Biosystems). RNA quantity and quality were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and A260/280 nm ≥1.8 was determined for quantitative analysis using the procedures described in previous studies (10,17,18).

TLDA. The miRNA expression profile of the L4/5 dorsal spinal cord of CCI rats was analyzed using TLDA Rodent MicroRNA Cards v.3 A and B (Applied Biosystems). We used the standard method that was evaluated in a previous study (10,17-19). Each card contains 375 preloaded rodent miRNA targets, all catalogued in the miRBase database, and three endogenous controls: Mamm U6, U87 and Y1. In this study, we used U87 as the endogenous normalizer. TLDAs were performed using a four-step process. The first step was the Megaplex RT Reaction in which 60 ng of total RNA per sample was reverse-transcribed using Megaplex RT primer Pool A and B, with up to 381 stem-looped primers per pool, and the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems). The next step was the preamplification reaction in which TaqMan PreAmp Master Mix, 2X and Megaplex PreAmp Primers (Applied Biosystems) were added to each complementary DNA (cDNA), and the reaction was performed. The preamplified product was diluted with 0.1X TE at pH8.0 (Wako, Tokyo, Japan). In the third step, each of the resulting cDNAs after the preamplification reaction were diluted with the TaqMan Universal PCR master mix (Applied Biosystems) and deionized distilled water (Wako), and were loaded into one of the eight fill ports on the TLDA microfluidic card. The card was centrifuged for 1 min at 331 x g to distribute samples to multiple wells connected to the fill ports and was 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).

Real-time RT-PCR data analysis was performed using DataAssist software v2.0 (Applied Biosystems). The resulting data are shown as 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. The expression level was calculated using the comparative Ct (ΔΔCt) method and was further analyzed by comparing the fold change relative to basal levels in the Day 0 sample. The results of the TLDA analysis were converted into a graphic display as a heat map based on hierarchical clustering using DataAssist version 2.0. Distances between samples and assays were calculated for hierarchical clustering based on ΔCt values using Euclidean Distance.

Statistical analysis. For statistical analyses of behavioral data, we used a two-tailed t-test and an ANOVA followed by Tukey's test. To compare the gene expression levels of sham-operated rats (Day 7, n=6; Day 14, n=6) with TLDA dates in the L4/5 dorsal spinal cords of CCI rats (Day 7, n=6; Day 14, n=6) within the same group, ANOVA was used followed by Tukey's test. All statistical procedures were performed using KyPlot 5.0 (KyensLab, Inc., Tokyo, Japan). All values are expressed as the means ± standard deviation. A P-value <0.01 was considered to indicate statistically significant differences.

Results

Behavioral tests. Compared with the values from sham-operated rats, the latencies of paw withdrawal thresholds in response to mechanical stimulation (Fig 2A) and paw withdrawal from thermal stimulation (Fig 2B) on the ligated ipsilateral side in CCI rats were significantly decreased at Day 1 after surgery. The hypersensitivity peaked at 7 days, and was then maintained at the same level until Day 13 after surgery (P<0.01) (Fig. 2).

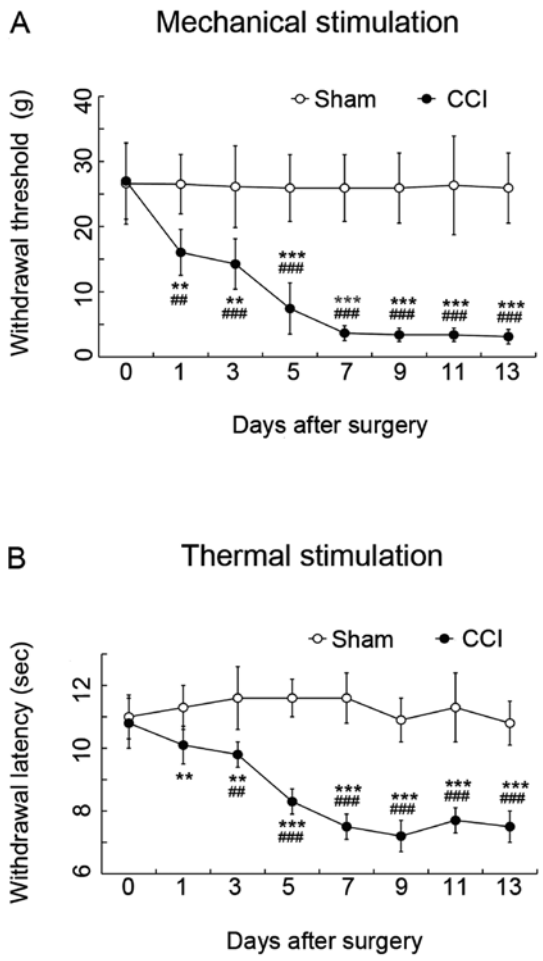

Figure 2: Behavioral tests for pain assessment. The effect of nerve injury on (A) mechanical allodynia and (B) thermal hypersensitivity of the ligated ipsilateral side and control contralateral side in the rat model are shown. Asterisks (*) indicate a significant difference in the measured values for the ligated side compared with those for the contralateral side on the same day using a paired *t*-test (**P<0.01, ***P<0.001), and pound symbols (#) indicate a significant difference in the measured values on each day compared with the preoperative values (Day 0) using Tukey's multiple comparison test ($^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001). Values are means ± standard errors of the mean.

TLDA analysis. Using the TLDA card, 375 out of 750 miRNAs were peculiarly expressed in the rat tissue. In CCI rats (Day 7 and/or Day 14), 111 of 375 miRNAs were significantly regulated compared with sham-operated rats (Day 7 and/or Day 14). The expression of 21 miRNAs (up, 8 miRNAs; down, 13 miRNAs) was significantly altered only in Day 7 CCI rats compared to Day 7 sham-operated rats. The expression of 65 miRNAs (up, 20 miRNAs; down, 45 miRNAs) was significantly altered only in Day 14 CCI rats compared to Day 14 sham rats. In the Day 7 and Day 14 groups, 25 miRNAs significantly changed expression (up, 3 miRNAs; down, 22 miRNAs). Details of the results are shown in Table I. We illustrated a clustergram of the samples and the significant differentially expressed miRNAs in the dorsal horn of the spinal cord as a heat map (Fig. 3). Heat maps are commonly used for visualization of high-dimensional data on a two-dimensional image with colors representing the intensity values. Heat maps are typically used in gene expression analysis to represent the level of expression of many genes across a number of comparable samples. After individually clustering columns (samples) and rows (miRNAs), the heat map simultaneously displays the separate samples and miRNA clustering in one graphic. Red and blue colors indicate relatively high and low expression, respectively. The dendrogram at the top of the figure indicates the relatedness of the samples based on overall miRNA expression values. The dendrogram on the left side of the figure orders miRNAs into groups based on the divergence of miRNA expression values among the samples. In the top dendrogram of the heat map, these 111 miRNAs separate three branches, which are the sham group (Day 7 and Day 14), the Day 7 CCI group and the Day 14 CCI group. Sham samples from Day 7 and Day 14 rats are not separated from each other. These results demonstrated no difference in the expression pattern between Day 7 and Day 14 sham-operated rats, and the role of miRNAs in the dorsal horn of the spinal cord of CCI rats is changed with time.

Text mining of miRNA associations with gene function. Next, we sought to identify the role of miRNA changes in this study by text mining using PubMed. In 111 miRNAs, there were 76 (68.5%) miRNAs analyzed in previous reports and 36 miRNAs (32.4%) played a role related to nerve development and maintenance (14 miRNAs), tumors of the nervous system (18 miRNAs), and neurodegenerative diseases (8 miRNAs). Four miRNAs overlapped. There were three miRNAs related to neuropathic pain; miR-500, -221 and -21 (20,21).

Table I. The varied expression of microRNAs (miRNAs) on chronic constrictive injury (CCI) rats compared to the sham rats without ligated sciatic nerve.

Assay	Fold change ± standard deviation (SD) CCI7 / CCI14	Sham7 / Sham14	P-value
miRNAs downregulated in both the Day 7 and Day 14 groups			
hsa-miR-22#	1.134±0.098	0.387±0.062	$1.74x10^{-6}$
	0.757±0.224	0.440±0.068	0.00172
mmu-miR-496	1.231±0.259	0.227±0.119	$1.74x10^{-6}$
	1.312±0.204	0.078±0.042	$1.74x10^{-6}$
mmu-miR-151-3p	1.156±0.262	0.310±0.095	$2.32x10^{-6}$
	0.899±0.236	0.468±0.158	0.00581
mmu-miR-24-2#	0.883±0.099	0.500±0.075	$9.80x10^{-6}$
	0.803±0.102	0.384±0.072	$3.52x10^{-6}$
mmu-miR-324-5p	0.994±0.195	0.398±0.096	$8.50x10^{-5}$
	0.957±0.300	0.467±0.056	0.00102
rno-miR-345-3p	0.956±0.159	0.289±0.216	0.000157
	1.261±0.313	0.181±0.039	$1.80x10^{-6}$
mmu-miR-127	1.269±0.174	0.676±0.116	0.000162
	1.313±0.329	0.632±0.124	$2.37x10^{-5}$
mmu-miR-125b-5p	1.057±0.202	0.454±0.075	0.000262
	1.034±0.345	0.494±0.128	0.00100
mmu-miR-221	1.360±0.224	0.519±0.205	0.000334
	1.334±0.519	0.391±0.109	$7.29x10^{-5}$
mmu-miR-296-5p	1.531±0.388	0.710±0.085	0.000337
	1.250±0.404	0.618±0.212	0.00603
rno-miR-377	0.967±0.165	0.158±0.087	0.000416
	1.425±0.570	0.090±0.052	$1.91x10^{-6}$
mmu-miR-365	1.492±0.387	0.276±0.238	0.000918
	1.681±0.906	0.028±0.035	$1.59x10^{-5}$
mmu-miR-598	1.083±0.085	0.577±0.147	0.00104
	1.259±0.318	0.664±0.144	0.000139
mmu-miR-7a	0.996±0.215	0.485±0.152	0.00206
	1.093±0.231	0.623±0.202	0.00486
mmu-miR-101b	1.002±0.119	0.690±0.135	0.00319
	1.070±0.149	0.452±0.107	$1.90x10^{-6}$
mmu-miR-29b	1.004±0.202	0.481±0.187	0.00324
	1.009±0.278	0.396±0.143	0.000553
rno-miR-336	0.976±0.203	0.099±0.088	0.00362
	1.500±0.761	0.057±0.030	$6.75x10^{-6}$
hsa-miR-493-3p	0.794±0.156	0.373±0.153	0.00384
	1.204±0.244	0.420±0.083	$2.44x10^{-6}$
mmu-miR-322	1.068±0.196	0.641±0.146	0.00469
	1.016±0.256	0.531±0.112	0.00123
mmu-miR-21	1.400±0.084	0.612±0.119	0.00677
	1.887±0.747	0.718±0.179	$6.76x10^{-5}$
mmu-miR-27b	1.018±0.111	0.545±0.126	0.00687
	1.220±0.391	0.620±0.137	0.000534
rno-miR-632	1.078±0.177	0.571±0.260	0.00901
	1.163±0.360	0.324±0.087	$2.30x10^{-5}$

Table I. Continued.

Assay	CCI7	Sham7	P-value
miRNAs downregulated only in the Day 7 group			
mmu-miR-193#	1.014±0.058	0.368±0.062	$1.74x10^{-6}$
hsa-miR-29a#	0.984±0.193	0.448±0.159	0.000410
mmu-miR-19b	1.279±0.195	0.657±0.167	0.000139
rno-miR-339-3p	1.046±0.178	0.464±0.246	0.000662
mmu-miR-106b	0.985±0.187	0.408±0.124	0.000213
mmu-miR-500	1.194±0.208	0.534±0.130	0.000216
mmu-miR-375	1.667±0.438	0.800±0.314	0.00113
hsa-miR-378	0.914±0.052	0.487±0.235	0.00142
mmu-miR-28	0.934±0.170	0.478±0.079	0.00172
mmu-miR-30b	1.201±0.180	0.775±0.125	0.00278
mmu-miR-381	1.157±0.380	0.397±0.349	0.00390
mmu-miR-337	0.974±0.106	0.572±0.114	0.00581
mmu-miR-203	0.999±0.223	0.491±0.169	0.00906
Assay	CCI7 CCI14	Sham7 Sham14	P-value
miRNAs upregulated in both the Day 7 and Day 14 groups			
mmu-miR-539	1.077±0.279	1044±240.5	$1.74x10^{-6}$
	0.840±0.222	949.9±201.6	$1.74x10^{-6}$
rno-miR-381	1.192±0.157	7.123±1.091	$1.74x10^{-6}$
	1.733±0.458	6.369±1.251	$1.74x10^{-6}$
mmu-miR-323-3p	1.024±0.190	2.541±0.682	$3.29x10^{-5}$
	0.948±0.207	2.591±0.594	$1.09x10^{-5}$
Assay	CCI7	Sham7	P-value
miRNAs upregulated only in the Day 7 group			
mmu-miR-193	0.913±0.147	53.40±13.73	$1.74x10^{-6}$
rno-miR-489	0.850±0.230	16.59±1.874	0.000107
rno-miR-346	1.075±0.423	7.038±4.246	0.000256
rno-miR-409-5p	0.365±0.059	16.68±10.57	0.000315
hsa-miR-99b#	1.204±0.287	3.063±1.397	0.00117
hsa-miR-136	1.010±0.201	1.855±0.269	0.00228
mmu-miR-770-5p	1.160±0.159	2.202±0.319	0.00252
mmu-miR-376a	1.241±0.241	2.307±0.677	0.000343
Assay	CCI14	Sham14	P-value
miRNAs upregulated only in the Day14 group			
mmu-let-7d#	0.058±0.009	21.43±9.389	$1.97x10^{-6}$
mmu-miR-139-3p	0.335±0.271	3.056±0.900	$3.12x10^{-6}$
mmu-miR-296-3p	0.997±0.186	24.75±14.28	$1.70x10^{-5}$
mmu-miR-134	0.915±0.253	2.687±1.000	$2.88x10^{-5}$
rno-miR-219-1-3p	0.837±0.196	2.625±0.777	$3.76x10^{-5}$
mmu-miR-342-3p	1.284±0.237	5.354±2.611	$9.37x10^{-5}$
mmu-miR-126-3p	0.915±0.142	2.171±0.801	0.000226

Table I. Continued.

Assay	CCI14	Sham14	P-value
miRNAs upregulated only in the Day14 group			
mmu-miR-186	1.443±0.502	5.076±2.501	0.000289
mmu-miR-125a-3p	0.886±0.227	2.700±1.261	0.000328
mmu-miR-449a	1.874±0.577	6.181±3.405	0.00113
mmu-miR-191	1.092±0.312	2.671±1.134	0.00120
mmu-miR-764-5p	0.827±0.762	3.243±1.237	0.00123
mmu-miR-139-5p	0.951±0.195	2.003±0.781	0.00175
mmu-miR-146a	1.303±0.409	2.670±1.048	0.00179
mmu-miR-672	0.755±0.124	3.127±1.685	0.00211
mmu-miR-150	0.885±0.263	1.848±0.731	0.00246
mmu-miR-511	0.073±0.014	1.710±1.402	0.00329
mmu-miR-380-5p	1.066±0.347	1.833±0.412	0.00441
mmu-miR-187	0.861±0.281	1.657±0.507	0.00449
hsa-miR-124#	2.265±3.467	12.67±9.371	0.00688
Assay	CCI14	Sham14	P-value
miRNAs downregulated only in the Day 14 group			
mmu-miR-374-5p	1.570±0.363	0.291±0.191	$1.76x10^{-6}$
mmu-miR-137	1.487±0.231	0.688±0.153	$2.04x10^{-6}$
mmu-miR-27a	1.743±0.367	0.847±0.148	$2.94x10^{-6}$
rno-miR-505	1.720±0.443	0.743±0.072	$3.13x10^{-6}$
rno-miR-421	3.157±1.519	0.122±0.206	$4.06x10^{-6}$
rno-miR-29c#	1.317±0.283	0.674±0.105	$7.19x10^{-6}$
mmu-miR-369-5p	1.092±0.276	0.331±0.099	$1.06x10^{-5}$
mmu-miR-879	2.539±0.761	1.043±0.309	$1.17x10^{-5}$
mmu-miR-190	2.182±0.699	0.964±0.168	$2.58x10^{-5}$
mmu-miR-674#	0.948±0.187	0.526±0.098	$4.36x10^{-5}$
mmu-miR-487b	1.225±0.264	0.703±0.148	$4.33x10^{-5}$
mmu-miR-325	1.885±0.388	1.044±0.200	$4.43x10^{-5}$
mmu-miR-542-3p	1.455±0.388	0.649±0.232	$4.66x10^{-5}$
mmu-miR-96	1.170±0.310	0.526±0.145	$7.87x10^{-5}$
rno-miR-99a#	1.038±0.222	0.574±0.124	$8.45x10^{-5}$
mmu-miR-26b	1.635±0.333	0.995±0.149	0.000101
hsa-miR-27a#	1.159±0.182	0.423±0.236	0.000127
mmu-miR-32	1.708±0.243	0.871±0.358	0.000145
rno-miR-207	2.362±0.314	0.858±0.934	0.000188
hsa-miR-423-3P	1.093±0.156	0.709±0.182	0.000258
mmu-miR-196a#	1.600±0.582	0.520±0.233	0.000332
mmu-miR-130b	1.406±0.410	0.562±0.185	0.000342
hsa-miR-9#	1.104±0.252	0.619±0.129	0.000354
mmu-miR-335-5p	1.455±0.413	0.801±0.132	0.000374
mmu-miR-26a	1.663±0.443	0.965±0.161	0.000398
hsa-miR-136#	1.505±0.268	0.959±0.241	0.000656
hsa-miR-340	1.085±0.204	0.632±0.055	0.000769
rno-miR-504	0.969±0.166	0.586±0.170	0.000993
mmu-miR-130a	1.242±0.337	0.468±0.151	0.00153
hsa-miR-455	0.989±0.161	0.643±0.131	0.00157
mmu-miR-142-3p	1.974±0.970	0.730±0.300	0.00188
hsa-miR-26b#	1.233±0.226	0.781±0.201	0.00194

Table I. Continued.

Assay	CCI14	Sham14	P-value
miRNAs downregulated only in the Day 14 group			
rno-miR-450a	1.131±0.305	0.536±0.134	0.00257
mmu-let-7i	1.313±0.331	0.750±0.234	0.00292
rno-miR-532-5p	1.238±0.355	0.731±0.074	0.00297
mmu-miR-34c	1.439±0.615	0.691±0.181	0.00306
mmu-miR-129-5p	2.072±0.537	1.345±0.284	0.00342
mmu-miR-497	1.086±0.462	0.486±0.092	0.00360
mmu-miR-135a	1.936±0.384	1.294±0.340	0.00398
rno-miR-351	1.517±0.374	0.843±0.286	0.00468
mmu-miR-369-3 p	1.837±0.561	1.021±0.477	0.00516
mmu-miR-148b	1.526±0.398	0.996±0.195	0.00585
rno-miR-409-3P	1.442±0.260	0.930±0.378	0.00667
hsa-miR-140-3p	1.698±0.502	0.948±0.425	0.00691
mmu-miR-29c	1.748±1.390	0.399±0.110	0.00910

The table is presented based on P-value.

Discussion

In the present study, we comprehensively analyzed the dorsal horn of the spinal cord, which is an important organ for synaptic plasticity, pain control and treatment of neuropathic pain, in the CCI rat model using TLDA with the onset and maintenance of hyperalgesia. Comparison between sham-operated and CCI rats in the same group revealed expression changes in several miRNAs, as hypothesized. This was particularly true for regulated miRNAs, anticipated to be associated with the mechanisms underlying neuropathic pain. In the pain field, many reports on miRNA expression have been published since Bai *et al* (22) reported miRNA expression following muscle pain (7-10,23). However, the available data do not clarify the role of miRNA expression in the dorsal horn of the spinal cord in neuropathic pain. In this study, it is suggested, for the first time, that the expression of many miRNAs is regulated, and that the number of changed miRNAs increases with the passage of time after ligation of the sciatic nerve in the dorsal horn of the spinal cord in the neuropathic pain model, although hypersensitivity has been stable. In a previous study using mice, the expression of miRNA in the dorsal horn of the spinal cord was changed 14 days after sciatic nerve injury, later than the change in the dorsal root ganglion (7). We speculated that part of the role of the dorsal horn of the spinal cord may be to maintain the symptoms of neuropathic pain. Previous studies have identified a number of miRNAs expressed in the spinal cord (24-27). The miR-29a/b/c, -26a, -142-3p and -193 are highly expressed in the spinal cord. The miR-129, -146a and -21 have also been reported to play a role in the reorganization or recovery of the nervous system after injury and in the nervous system development, apoptosis and synaptic plasticity (28-31). Above all, miR-26a/b and miR-29 are strongly expressed in astrocytes, and regulate the role of astrocytes in the structure of the brain and spinal cord. They may also be involved in the expression of neurotransmitters such as glutamate transporters to modulate synaptic transmission and to repair the nervous system (24,25). It is of note that miR-28 was associated with μ-opioid receptors and/or expression of cAMP response element-binding 1 (CREB-1), which has a role in neuronal plasticity (32,33). In addition, miR-203 targets γ-aminobutyric acid (GABA)-A receptors, which are a class of receptors that respond to the neurotransmitter GABA, the chief inhibitory neurotransmitter in the vertebrate central nervous system (34). Although several miRNAs changed expression, their roles in neuropathic pain have yet to be analyzed. We speculate that numerous miRNAs play key roles in the molecular mechanisms responsible for nerve regeneration, synaptic plasticity or analgesia, similar to miR-26, miR-29, miR-28 and miR-203.

The comprehensive TLDA analysis performed in this study demonstrated changes in a large number of miRNAs in addition to those previously reported as being related to neuropathic pain. These findings are expected to contribute to our understanding of the role of miRNA in the spinal cord of patients with neuropathic pain and of the mechanisms underlying neuropathic pain and may be the first step towards the selection of effective therapeutic methods.

In this TLDA study, we report that the expression of many miRNAs is altered in the dorsal horn of the spinal cord in CCI rats, and we suggest the possibility that these changes play a role in the maintenance and development of, and therapy for, neuropathic pain. However, these data are not sufficient to make strong conclusions on the role of miRNA changes in neuropathic pain. In the study of miRNA biology, it is critical to predict changes in the target mRNA using available online target prediction software such as miRecords, TargetScan and PicTar, and to prove that a miRNA can bind to a target mRNA using a luciferase reporter assay. For example, it would be of benefit to clarify the role of mRNA coding mediators in the activation of microglia or in the control of analgesia, sero-

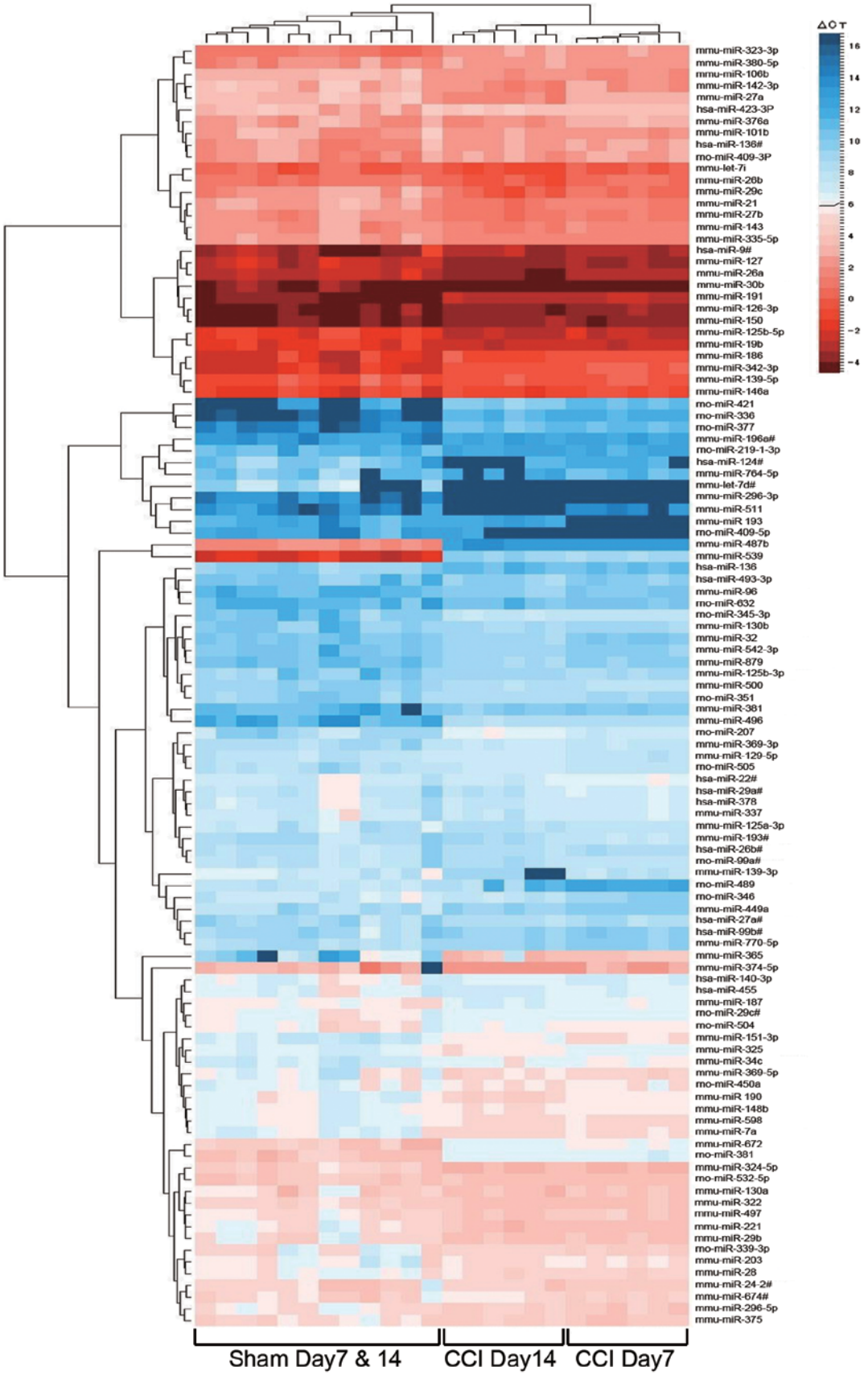

Figure 3. Heat map of the results of TaqMan® Low Density Array (TLDA) analysis. Heat maps show the microRNA (miRNA) expression pattern in the L4/5 dorsal spinal cord in Day 7 and Day 14 rats [chronic constrictive injury (CCI) vs. sham rats]. The distance measured is Euclidean Distance, and the clustering method is complete linkage. Red and blue colors indicate relatively high and low expression, respectively. Heat map shows the expression of the 111 miRNAs on TLDA that showed significantly regulated expression between CCI (n=6) and sham (n=6) rats in the Day 7 and Day 14 groups. Dendrograms of clustering analysis for miRNAs and samples are displayed on the top and left, respectively. The left dendrogram separates the expression profiles of the sham group (Day 7 and Day 14) from the CCI Day 7 and Day 14 groups.

tonin, adrenaline and acetylcholine. Identification of the target gene of these miRNAs should help to elucidate mechanisms underlying neuropathic pain and identify promising targets for future research.

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