[**Original Research**]

Neuropathic Pain-Like Stimuli Change the Expression of Ribosomal Proteins in the Amygdala: Genome-Wide Search for a "Pain-Associated Anxiety-Related Factor"

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Abstract: Neuropathic pain leads not only to increased chronic pain sensation but also to emotional deficits associated with this pain. These phenomena are considered to be able to explain the possible change in microendophenotypes related to the brain dysfunction associate with neuropathic pain-like stimuli. However, the molecular mechanism of such pain-induced emotional dysfunction has not yet been clarified. In a behavioral study, we observed the induction of pain-induced anxiogenic-like behavior as well as allodynia after 4 weeks of sciatic nerve ligation in BALB/c mice, whereas sciatic nerve ligation for 1 week induced only allodynia without anxiogenic-like behavior. We next performed a genome-wide analysis of the changes in the expression of mRNA and microRNA (miRNA) in the amygdala, which is the brain region that is considered to play a role in pain-associated emotional dysfunction, of mice with sciatic nerve ligation. In this genome-wide-expression study, we found that sciatic nerve ligation for both 1 and 4 weeks changed the expression of many of mRNAs and miRNAs in the amygdala. Based on a pathway analysis, sciatic nerve ligation for 4 weeks changed the expression of mRNAs and miRNAs that are related to several ribosomal proteins in the amygdala, compared to sciatic nerve ligation for 1 week. These results suggest that changes in the expression of ribosomal proteins in the amygdala may, at least in part, correspond to emotional deficits associated with pain sensation under a neuropathic pain-like state.

Key words: neuropathic pain, emotional deficit (anxiety), amygdala, microRNA, ribosomal protein

INTRODUCTION

Pain is a complex sensory and emotional experience. It can be classified as nociceptive pain, neuropathic pain, or nonorganic pain. Among these, neuropathic pain is the most difficult to treat in the pain clinic. Long-lasting pain leads not only to increased chronic pain but also to cognitive and emotional deficits that are comorbid with the pain[1]. These emotional disorders after nerve injury suggest that neuropathic pain-like stimuli may affect the upper central nervous system. It has been proposed that emotion can be controlled by mesolimbic systems such as the nucleus accumbens and amygdala. It is also documented that the functional activity of the amygdala is regulated by the activity of the prefrontal cortex (PFC) including the anterior cingulate cortex (CG), which is the terminus of the ascending pain pathway[2, 3]. Subsequently, it projects to some brain regions that are associated with the descending pain pathway and terminates in the dorsal raphe nucleus and periaqueductal gray matter[4]. It has been recognized that the amygdala is the brain region that is most closely related to pain-induced emotional dysfunction. In a previous study, we demonstrated that chronic pain eventually induced emotional disorder, such as increased anxiety, along with changes in brain activity[5]. However, the mechanism of pain-induced emotional dysfunction at the molecular level has not yet been clarified. On the other hand, there is a growing body of evidence that genome-wide studies could be useful for elucidating the mechanisms of diseases.

MicroRNAs (miRNAs), which are small (17–24 nucleotides), noncoding RNA molecules that direct the post-transcriptional suppression of gene expression, are believed to play an important role in regulating synaptic plasticity. miRNAs have been shown to be associated with cancer, cardiac disease, metabolic disease and so on[6–9]. In the field of pain-related brain research, we previously reported that neuropathic pain-like stimuli

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dramatically decreased the expression of miRNA200/429 in the nucleus accumbens and subsequently increased the protein level of its target protein, DNMT3a, which is a DNA methyltransferase (Imai et al., 2012)[10]. These findings suggest that neuropathic pain-like stimuli affect the "mesolimbic motivation/valuation circuitry". However, there have been few, if any, miRNA studies under a persistent neuropathic pain-like state with emotional disorders. In this study, we performed a genome-wide analysis on the changes in the expression of mRNA and miRNA in the amygdala of mice with nerve injury.

MATERIALS AND METHODS

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan, and was approved by the Animal Research Committee of Hoshi University.

1. Animals

Male BALB/c mice (7–8 weeks old) (Tokyo Laboratory Animals Science Co., Ltd.) were housed with *ad libitum* access to food and water and maintained on a 12 h light/dark cycle.

2. Neuropathic pain model

We produced a partial sciatic nerve ligation model by tying a tight ligature with 8–0 silk suture ×1/3 to 1/2 the diameter of the sciatic nerve on the right side (ipsilateral side) of mice under anesthesia with 3% isoflurane as described previously (Malmberg and Basbaum, 1998)[11]. In sham-operated mice, the nerve was exposed without ligation. We refer to these animals as 'ligation' or 'sham' animals in this study. This model may mimic important characteristics of chronic neuropathic pain in patients following peripheral nerve injury.

3. Mechanical allodynia

To quantify the sensitivity to a tactile stimulus, paw withdrawal in response to a tactile stimulus was measured using a bending force (0.16 g) applied by von Frey filaments (North Coast Medical, Inc., Morgan Hill, CA, USA) (Narita et al., 2005)[12]. A von Frey filament was applied to the plantar surface of each hind paw for 3 s, and this was repeated three times with an inter-trial interval of at least 5 s. Paw withdrawal in response to a tactile stimulus was evaluated by scoring as follows: 0, no response; 1, a slow or slight response to the stimulus; 2, a quick withdrawal response away from the stimulus without flinching and/or licking; 3, an intense withdrawal response away from the stimulus with brisk flinching and/or licking. The paw withdrawal in response to each filament was determined as the average of two scores per paw. Paw movements associated with locomotion or weight-shifting were not counted as a response. Before the behavioral responses to tactile stimuli were tested, the mice were habituated for at least 30 min on an elevated nylon mesh floor. Under these conditions, paw withdrawal in response to a tactile stimulus was tested. All animals received just one treatment. All behavioral experiences were carefully measured in a non-blinded fashion.

4. Elevated plus maze

We used the elevated plus-maze test to measure anxiogenic-like behaviors. This test has been used extensively to identify novel anxiolytic agents and to investigate the physiological and neurochemical basis of anxiety (Dawson and Tricklebank, 1995)[13]. The elevated plus-maze consisted of two opposing open ($30 \times 6 \times 0.3$ cm^2 each) and closed arms ($30 \times 6 \times 15$ cm^2 each) joined by a common central platform (mice, 9×9 cm^2). The maze was elevated 40 cm above the floor. The open and closed arms and the central platform were subjected to approximately equal illumination. Arm entry and exit were defined as all four paws into and out of an arm, respectively. Entries into the open and closed arms were also scored. Mice were used for this procedure at 7 days and 28 days after nerve ligation.

5. Brain sample preparation for *in vitro* analysis

As previously described (Narita et al., 2001)[14], after mice were killed and the whole brain was removed, thick coronal sections of the brain, including the amygdala (−0.9 to −2.8 mm from bregma), were initially dissected using Brain Blocker (NeuroScience, Inc.) on an ice-cold glass plate. The amygdala was then extracted from coronal sections. Brain positions were determined according to an atlas of the mouse brain (Franklin and Paxinos, 1997)[15].

6. Micro RNA and mRNA expression profiling

Total RNA, including miRNAs, was extracted from the amygdala of mice using the mirVana miRNA Isolation Kit (Applied Biosystems, Inc.). mRNA expression profiles were determined using a GeneChip Mouse Gene 2.0 ST Array (Filgen, Inc.). Differentially expressed genes were defined as genes that showed at least a 1.5-fold change. miRNA expression profiles were determined using a GeneChip miRNA 4.0 Array (Filgen, Inc.). Differentially expressed miRNAs were defined as those that showed at least a 2-fold change. Data analysis was performed with GeneSpring GX software (Agilent Technologies, Inc.). The analysis was divided into 3 parts: a genome-wide comparative analysis of changes in expression 1 week after sciatic nerve ligation vs. sham operation (Factor 1), a genome-wide comparative analysis of changes in expression 4 weeks after sciatic nerve ligation vs. sham operation (Factor 2), a genome-wide comparative analysis of changes in expression 1 week after sciatic nerve ligation vs. 4 weeks after sciatic nerve ligation (Factor 3).

7. Micro RNA and mRNA pathway analysis

The CEL file of the miRNA summarized by Affymetrix Expression Console™ Software and then normalized was processed using GeneSpring under the following conditions: Shift to 75th percentile, Baseline transformation: median of all samples. Furthermore, we extracted miRNAs that showed a 1.5 or 2-fold change in expression, either up or down. Subsequently, target genes were searched (Target Scan: http://www.targetscan. org/), and

we extracted mRNAs that showed a 1.2 or 1.25-fold change, either up or down. On the other hand, the CEL file of the mRNA summarized using GeneSpring by the algorithm exonRMA16 and then normalized was processed under the following conditions: Quantile, Baseline transformation: median of all samples. For a biological function analysis, we used gene ontology (GO) term enrichment analysis and Wiki pathway mapping through GeneSpring.

8. Statistical data analysis

Data are expressed as the mean with SEM. The statistical significance of differences between groups was assessed with Mann-Whitney *U* test. All statistical analyses were performed with Prism version 5.0c (GraphPad Software).

RESULTS

There was no difference in the anxiogenic-like behavior or the latency of paw withdrawal between sham-operated and sciatic nerve-ligated mice before surgery. The withdrawal latencies of the ipsilateral paw in response to the tactile stimuli were dramatically decreased at both 1 and 4 weeks after ligation compared to sham operation (Fig. 1-a). Under these conditions, anxiogenic-like behavior was evaluated. In the elevated plus-maze test, the time spent in the open arms was dramatically decreased in sciatic nerve-ligated mice at 4 weeks after surgery compared to sham operation, whereas it was not changed within 1 week after ligation (Fig. 1-b). On the other hand, the number of total entries, as a general activity-related parameter, wasn't significantly changed by sciatic nerve ligation for 4–6 weeks after surgery (22.8 ± 4.7 times) compared to sham operation (32.4 ± 4.8 times). These results suggest that neuropathic pain-like stimuli eventually lead to a long-lasting pain sensation along with anxiety in mice.

In the gene expression study, we found that sciatic nerve ligation for 1 week induced the up-expression of 494 miRNAs and 133 mRNAs, and the down-expression of 466 miRNAs and 4 mRNAs compared to sham operation (Factor 1). Sciatic nerve ligation for 4 weeks resulted in the up-expression of 506 miRNAs and 304 mRNAs, and the down-expression of 1,770 miRNAs and 269 mRNAs compared to sham operation (Factor 2). Furthermore, it produced the up-expression of 606 miRNAs and 375 mRNAs, and the down-expression of 2,286 miRNAs and 300 mRNAs compared to sciatic nerve ligation for 1 week (Factor 3). Taken together with the behavioral results, we defined the "Factor 3" as the "pain-associated anxiety-related factor". At the intersection of "Factor 1" and "Factor 2", 55 miRNAs and 1 mRNA showed up-expression, and 146 miRNAs and no mRNAs showed down-expression. The intersection of "Factor 1" and "Factor 3" included 1 miRNA and 1 mRNA that showed up-expression, and 110 miRNAs and no mRNAs that showed down-expression. The intersection of "Factor 2" and "Factor 3" consisted of 260 miRNAs and 150 mRNAs that showed up-expression, and 1,241 miRNAs and 104 mRNAs that showed down-expression. At the intersection of all 3 factors, there were 38 miRNAs and 6 mRNAs that showed up-expression, and 34 miRNAs and no mRNAs that showed down-expression (Fig. 1-c, d).

In a hierarchical clustering analysis, sciatic nerve ligation for 4 weeks in "Factor 2" induced the most dramatic change in miRNAs (Fig. 3-a) and mRNAs (Fig. 2-a) in the amygdala. As a result of GO (Table 1) and the pathway (Fig. 2-b, Table 2) analysis for mRNA, several ribosomal proteins were dramatically changed in "Factor 2". Furthermore, the target genes of the miRNAs that were changed in "Factor 2" included these ribosomal proteins (Fig. 3-b, Table 3). In a hierarchical clustering analysis, there were several miRNAs (Fig. 5-a) and mRNAs (Fig. 4-a) in "Factor 3". As a result of GO (Table 4) and the pathway (Fig. 4-b, Table 5) analysis for mRNAs, several ribosomal proteins were dramatically changed in "Factor 3". Furthermore, the target mRNAs of the miRNAs that were changed in "Factor 3" included 60S ribosomal protein L36 (Rpl36) (Fig. 5-b, Table 6).

DISCUSSION

In the present study, sciatic nerve ligation for 1 week produced a persistent painful state, but not anxiogenic-like behavior, in BALB/c mice, which are known to display high levels of anxiety[16]. On the other hand, sciatic nerve ligation for 4 weeks in this strain induced not only a persistent painful state but also anxiogenic-like behavior. This is consistent with our previous findings that sciatic nerve ligation for 1 week produced only a persistent painful state in C57 black mice, whereas sciatic nerve ligation for 4 weeks induced both a persistent painful state and anxiogenic-like behavior in this strain[5]. These findings suggest that long-lasting neuropathic pain-like stimuli lead not only to sustained pain but also to emotional deficits that are comorbid with the neuropathic pain.

In a previous study, we found that a single microinjection of a selective serotonin reuptake inhibitor into the amygdala prevented anxiogenic-like behaviors associated with sustained neuropathic pain, while it failed to relieve the pain itself. On the other hand, when injected into the primary somatosensory cortex of mice with sciatic nerve ligation, the same drug inhibited pain sensation, but did not show an anxiolytic effect[5]. When we considered these results along with the present findings, we hypothesized that the amygdala is one of the brain regions that plays a crucial role in the expression of neuropathic pain-associated anxiety.

To investigate the molecular mechanism of pain-induced emotional dysfunction, we next performed agenome-wide analysis of the changes in the expression of mRNA and miRNA in the amygdala of BALB/c mice with sciatic nerve ligation. In this genome-wide-expression study, we found that sciatic nerve ligation changed the expression of many mRNAs and miRNAs in the amygdala. Since our behavioral study indicated that

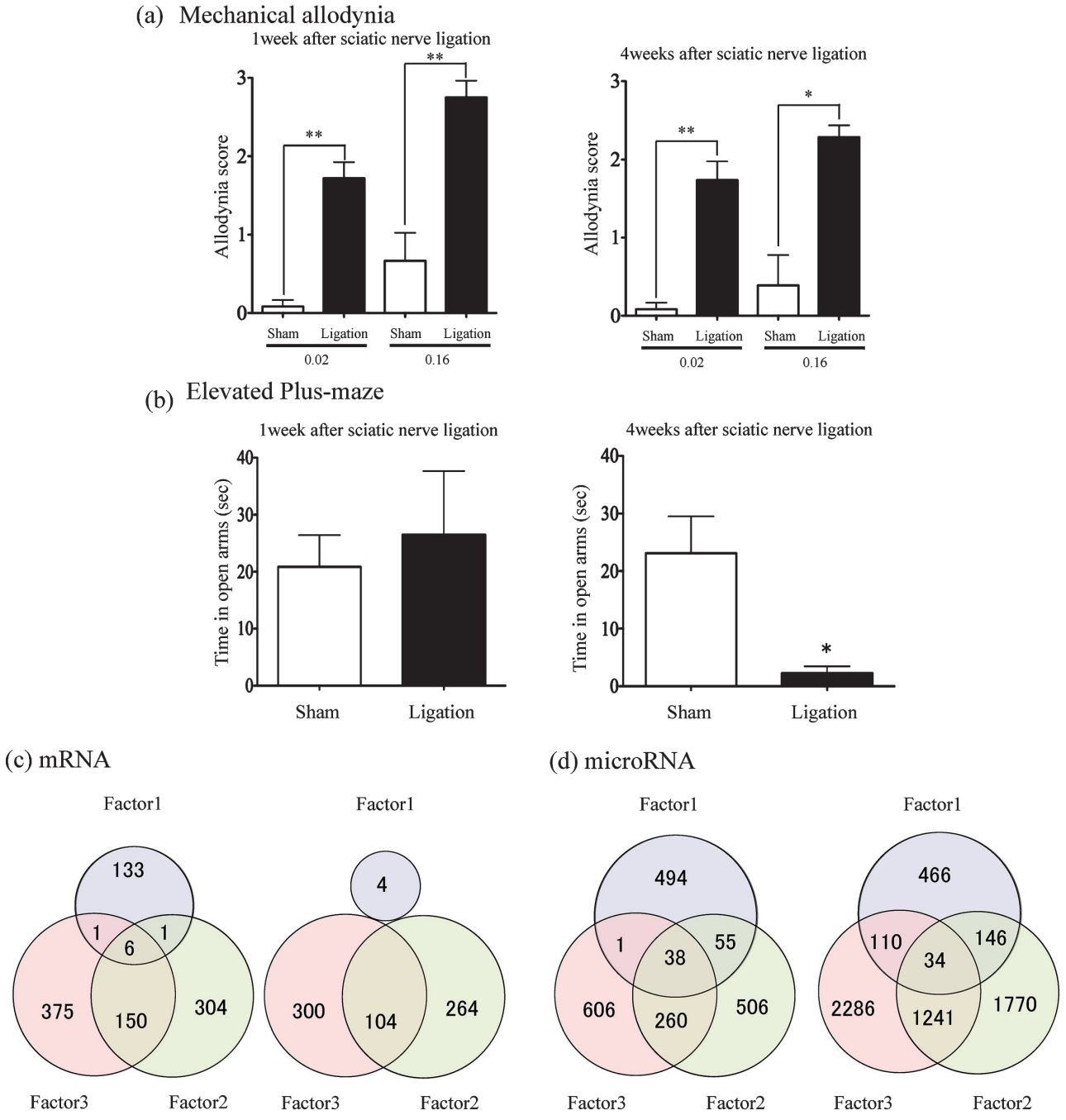
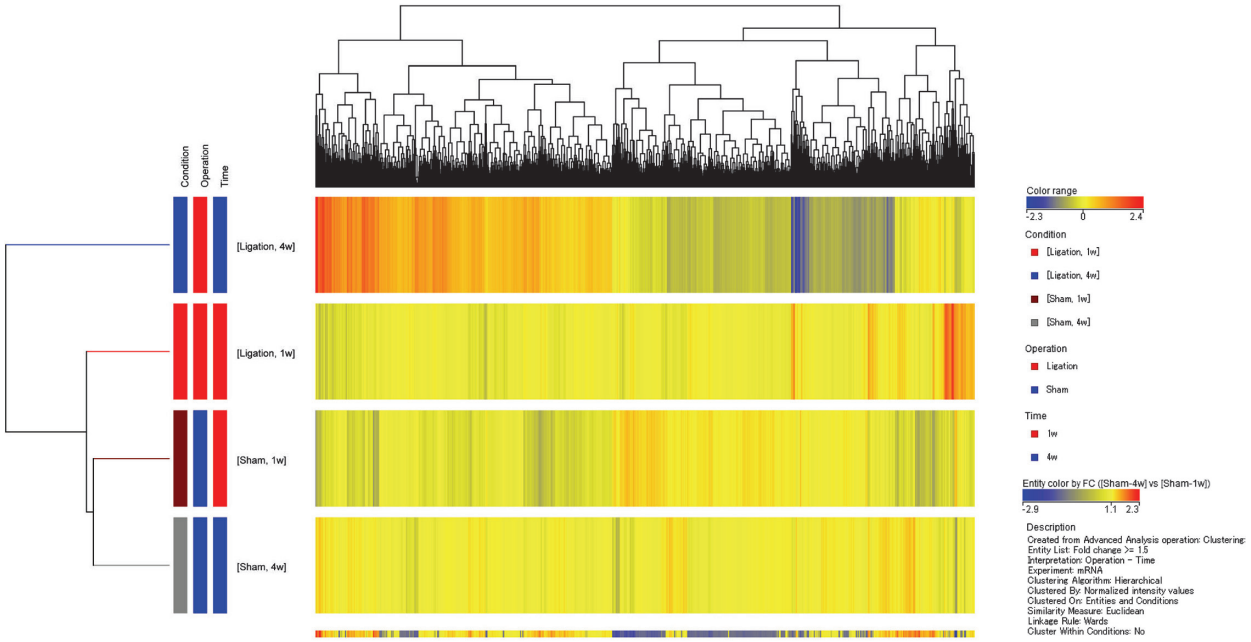


Fig. 1 (a) Changes in the latency of paw withdrawal after mechanical stimulation induced by sciatic nerve ligation in BALB/c mice. The data represent the results of paw withdrawal in response to a tactile stimulus on the ipsilateral side at 1 or 4 weeks after surgery. The tactile stimulus was applied using filaments with a bending force of 0.02 g or 0.16 g. (b) Time spent in the open arms in the elevated plus-maze was significantly decreased by sciatic nerve ligation at 4 weeks after surgery, but not at 1 week. Each point represents the mean ± *SEM* of 6–12 mice. $^{*}p<0.05$ and $^{**}p<0.01$ vs. sham-operated mice. (c, d) Venn diagram illustrating the commonly and exclusively expressed mRNA (c) and miRNA (d) between sciatic nerve ligation and sham-operated mice. "Factor 1" represents the data with sciatic nerve ligation for 1 week vs. sham operation for 1 week. "Factor 2" shows the data with sciatic nerve ligation for 4 weeks vs. sham operation for 4 weeks. "Factor 3" represents the data for sciatic nerve ligation for 4 weeks vs. sciatic nerve ligation for 1 week (fold change >2).

(a) Hierarchical clustering (mRNA)



(b) Cytoplasmic Ribosomal Proteins

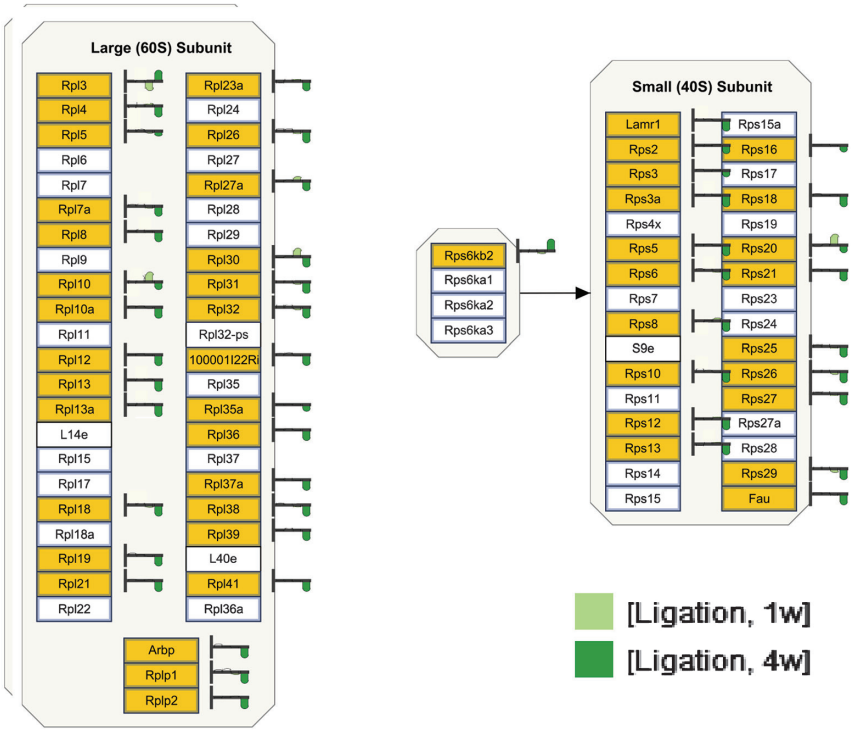


Fig. 2 (a) mRNA expression profiles in the amygdala of nerve-injured mice at 1 or 4 weeks vs. sham-operated mice (fold change >2). (b) Changes in mRNAs of ribosomal proteins in the amygdala of nerve-injured mice at 1 or 4 weeks. Yellowish green and green bars represent sciatic nerve-ligated amygdala for 1 week and 4 weeks vs. sham-operated mice, respectively. Each colored symbol "on the bar" represents "Up-expression", while those "under the bar" show "Down-expression" (fold change >1.5).

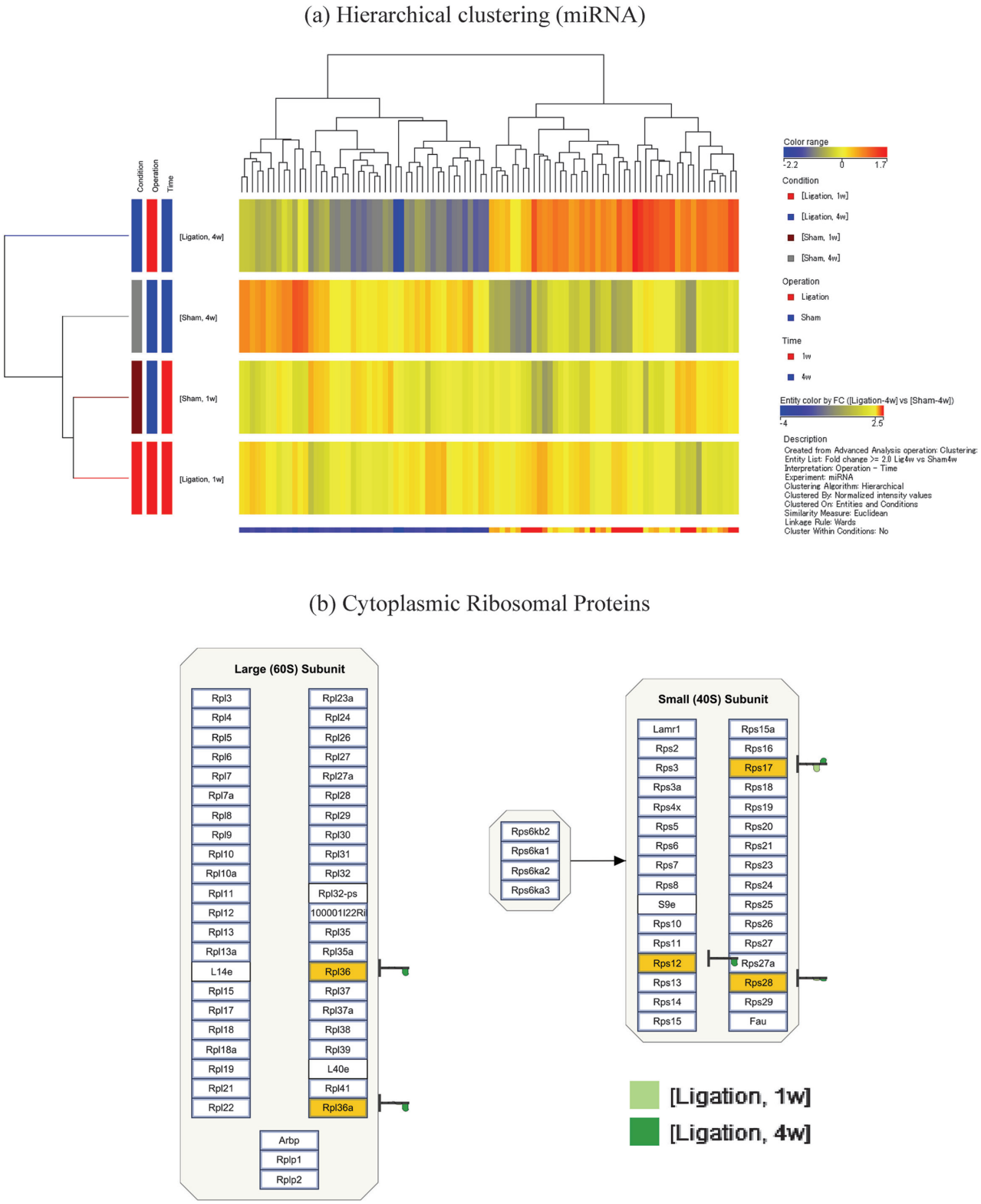


Fig. 3 (a) miRNA expression profiles in the amygdala of nerve-injured mice at 1 or 4 weeks vs. sham-operated mice (fold change >2). (b) Changes in possible mRNAs of ribosomal proteins related to miRNAs that were changed in the amygdala of nerve-injured mice at 1 or 4 weeks. Yellowish green and green bars represent sciatic nerve-ligated amygdala for 1 week and 4 weeks vs. sham-operated mice, respectively. Each colored symbol "on the bar" represents "Up-expression", while those "under the bar" show "Down-expression" (fold change >1.25).

Table 1 Classification of the target genes by GO ("Factor 2")

GO term	Count in selection	Count in total	*p*-value
Ribosome	144	342	0
Ribosomal subunit	126	258	0
Translation	155	452	0
Cytosolic part	132	303	0
Structural constituent of ribosome	131	278	0
Ribonucleoprotein complex	203	814	0
Cytosolic ribosome	117	202	0
Structural molecule activity	152	646	4.2E-45
Cytosolic large ribosomal subunit	63	119	1.15E-42
Cytosolic small ribosomal subunit	53	82	1.94E-42

Top ten enrichments of specific biological processes in Gene Ontology criteria among resistant and susceptible genes are listed from "Factor 2" (sciatic nerve ligation for 4 weeks vs. sham operation for 4 weeks) by GeneSpring bioinformatics resources (ligation 4 weeks vs. sham 4 weeks >1.5 fold).

Table 2 Wiki pathways list target mRNA ("Factor 2")

Pathway	Matched entities	Pathway entities of experiment type	*p*-value (mRNA)
Cytoplasmic ribosomal proteins	49	80	1.04E-38
Electron transport chain	31	102	2.04E-10
Oxidative phosphorylation	19	59	1.2E-09
mRNA processing	63	549	4.46E-09
Proteasome degradation	16	59	4.13E-07
Translation factors	12	51	4.26E-05
miRNAs involved in DNA damage response	8	49	8.94.E-03
Diurnally regulated genes with circadian orthologs	8	48	1.01.E-02
Retinol metabolism	7	39	1.06.E-02
Glutathione metabolism	4	19	2.92.E-02

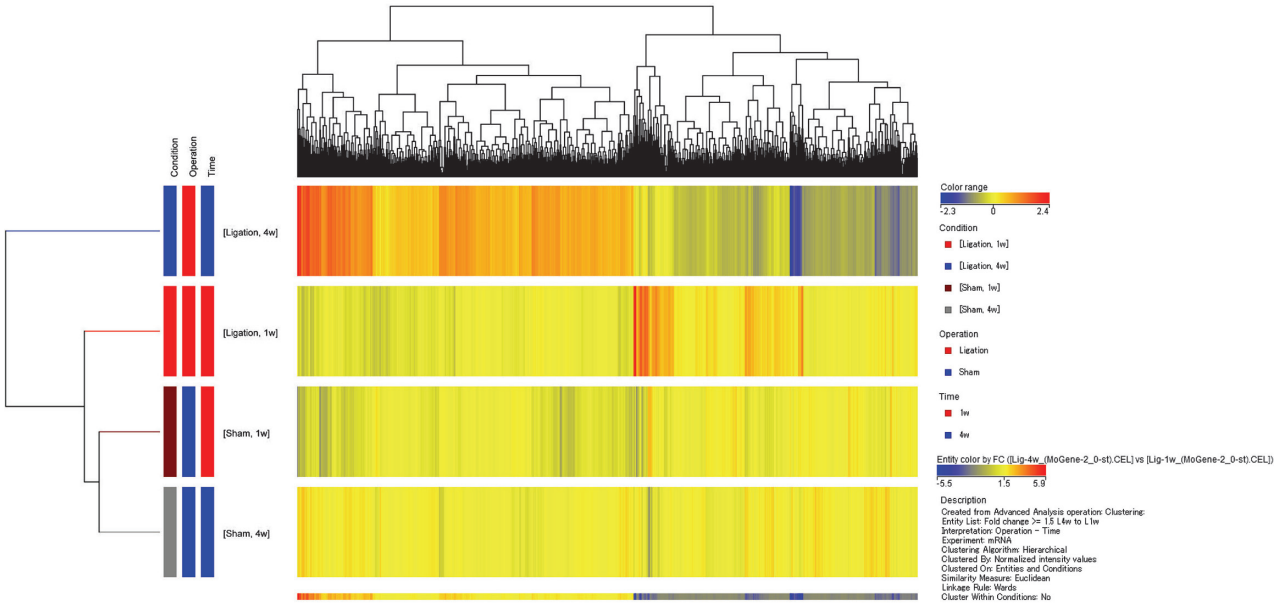
Top ten biofunctions predicted by pathways to be regulated by mRNA expression profiles in "Factor 2" are listed (ligation 4 weeks vs. sham 4 weeks >1.5 fold).

Table 3 Wiki pathways list target miRNA ("Factor 2")

Pathway	Matched entities	Pathway entities of experiment type	*p*-value
Cytoplasmic ribosomal proteins	5	80	2.09E-05
Electron transport chain	3	102	8.89.E-03
Amino acid metabolism	1	95	2.49.E-02
Phase I biotransformations, non P450	1	8	3.31.E-02
IL-5 signaling pathway	2	69	3.41.E-02
Purine metabolism	3	196	3.44.E-02
Serotonin and anxiety-related events	1	13	4.92.E-02

Biofunctions predicted by pathways to be regulated by mRNA expression profiles in "Factor 2" are listed (ligation 4 weeks vs. sham 4 weeks: miRNA >2 fold, mRNA >1.25 fold).

(a) Hierarchical clustering (mRNA)



(b) Cytoplasmic Ribosomal Proteins

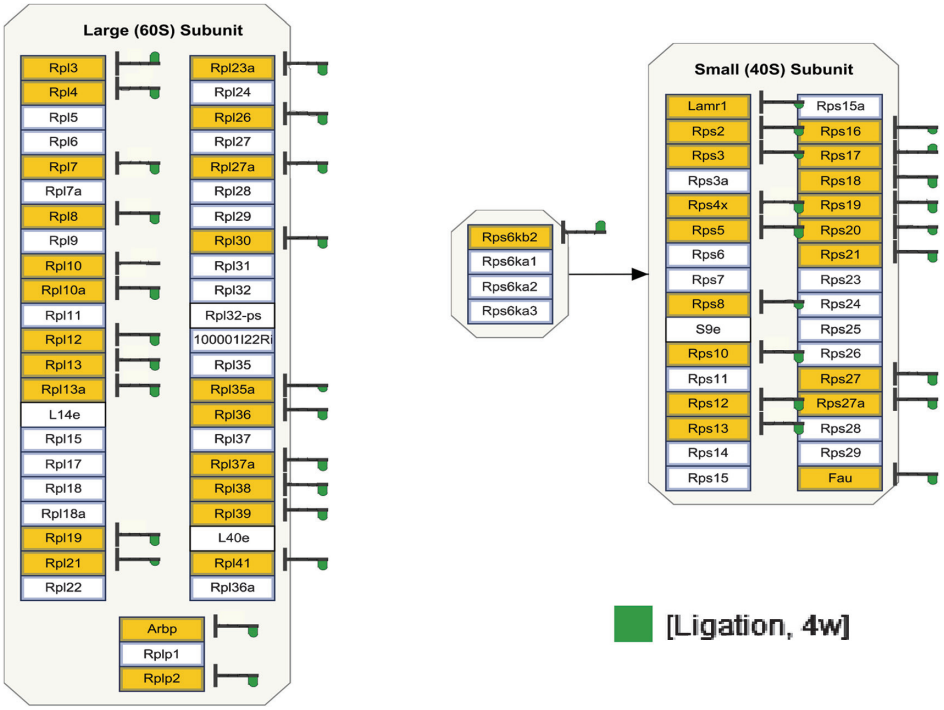


Fig. 4 (a) mRNA expression profiles in the amygdala of nerve-injured mice at 4 weeks vs. 1 week (fold change >1.5). (b) Changes in mRNAs of ribosomal proteins in the amygdala of nerve-injured mice at 4 weeks vs. 1 week. Green bar represents sciatic nerve-ligated amygdala for 4 weeks. Green symbol "on the bar" represents "Up-expression", while that "under the bar" shows "Down-expression" (fold change >1.5).

Table 4 Classification of the target genes by GO ("Factor 3")

GO term	Count in selection	Count in total	*p*-value
Structural constituent of ribosome	111	278	0
Ribonucleoprotein complex	160	814	0
Structural molecule activity	124	646	0
Ribosome	117	342	0
Cytosolic part	109	303	0
Ribosomal subunit	107	258	0
Translation	127	452	0
Cytosolic ribosome	100	202	0
Cytosolic large ribosomal subunit	55	119	1.7E-44
Large ribosomal subunit	59	155	1.04E-41

Top ten enrichments of specific biological processes in Gene Ontology criteria among resistant and susceptible genes are listed from "Factor 3" (sciatic nerve ligation for 4 weeks vs. the sciatic nerve ligation for 1 week) by GeneSpring bioinformatics resources (ligation 4 weeks vs. ligation 1 week >1.5 fold).

Table 5 Wiki pathways list target mRNA ("Factor 3")

Pathway	Matched entities	Pathway entities of experiment type	*p*-value (mRNA)
Cytoplasmic ribosomal proteins	42	80	6.69E-37
mRNA processing	56	549	3.31E-10
Electron transport chain	22	102	4.15E-10
Oxidative phosphorylation	13	59	4.99E-07
Translation factors	9	51	1.73E-04
Proteasome degradation	9	59	6.42E-04
Tryptophan metabolism	6	44	9.62.E-03
TNF-α NF-κB signaling pathway	15	184	1.08.E-02
Selenium micronutrient network	4	31	1.43.E-02
Fatty acid ω oxidation	2	7	3.19.E-02

Top ten biofunctions predicted by pathways to be regulated by mRNA expression profiles in "Factor 3" are listed (ligation 4 weeks vs. ligation 1 week >1.5 fold).

Table 6 Pathways list target miRNA ("Factor 3")

Pathway	Matched entities	Pathway entities of experiment type	*p*-value
One carbon metabolism	3	29	4.69.E-04
Estrogen signalling	4	74	6.31.E-04
p38 MAPK signaling pathway	3	34	7.53.E-04
Eukaryotic transcription initiation	3	41	1.22.E-03
MAPK signaling pathway	5	159	1.51.E-03
Apoptosis	3	83	9.59.E-03
MAPK cascade	2	29	1.01.E-02
Toll-like receptor signaling pathway	3	97	1.42.E-02
Oxidative damage	2	40	1.53.E-02
ESC pluripotency pathways	3	118	2.39.E-02
Notch signaling pathway	2	47	2.44.E-02
PodNet-protein-protein interactions in the podocyte	5	315	2.56.E-02
Translation factors	2	51	2.74.E-02
Fatty acid oxidation	1	11	5.63.E-02
Purine metabolism	3	196	5.97.E-02
Cytoplasmic ribosomal proteins	2	80	6.49.E-02

Biofunctions predicted by pathways to be regulated by mRNA expression profiles in "Factor 3" are listed (ligation 4 weeks vs. ligation 1 week: miRNA >1.5 fold, mRNA >1.2 fold).

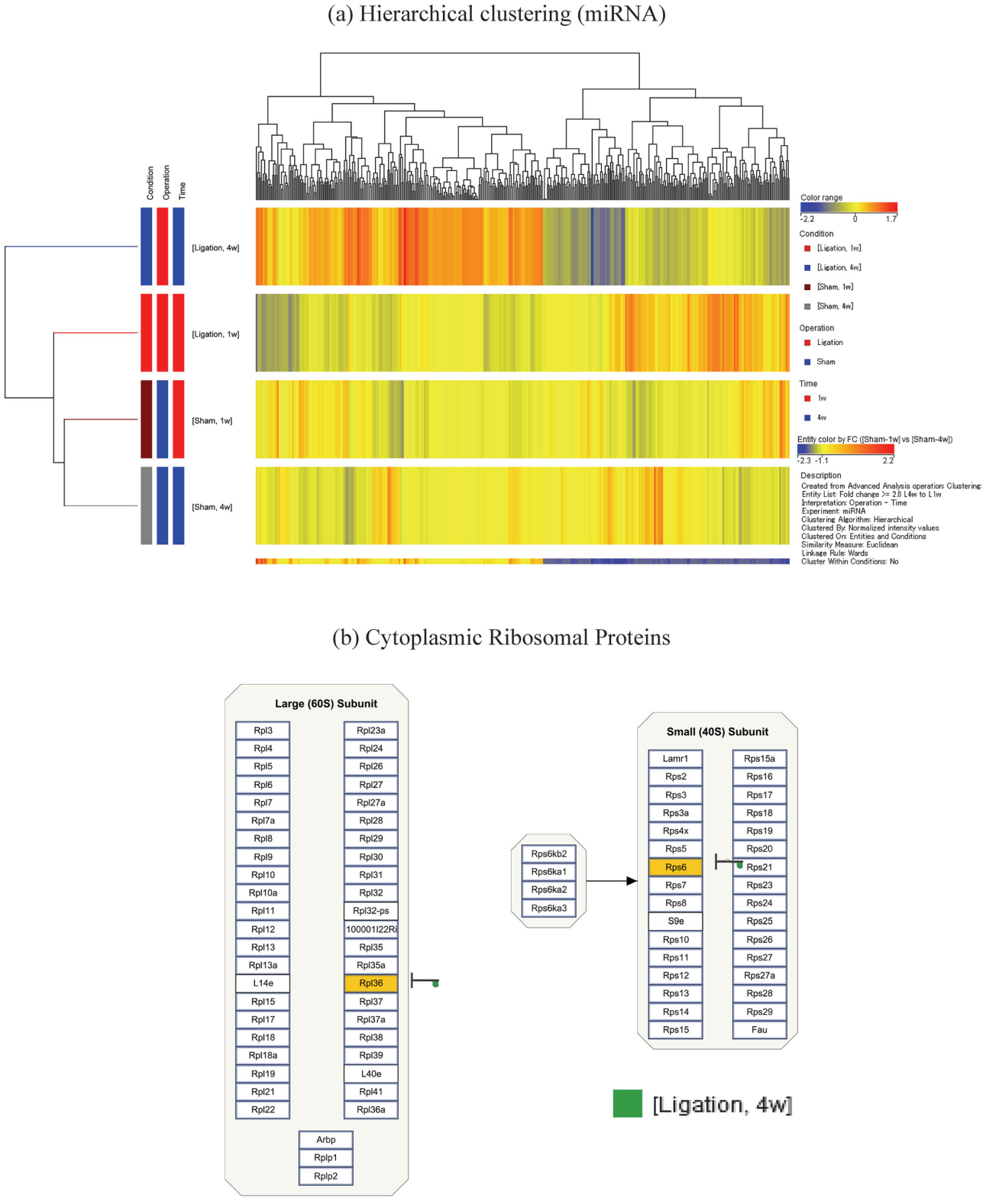


Fig. 5 (a) miRNA expression profiles in the amygdala of operated mice at 4 weeks vs. 1 week (fold change >1.5). (b) Changes in possible mRNAs of ribosomal proteins related to miRNAs that were changed in the amygdala of nerve-injured mice at 4 weeks. Green bar represents sciatic nerve-ligated amygdala for 4 weeks vs.1 week. Green symbol "on the bar" represents "Up-expression", while that "under the bar" shows "Down-expression" (fold change >1.2).

1 week of nerve injury produced only persistent pain, while ligation for 4 weeks induced both pain and anxiety, we mostly focused on the differences in gene expression between 1 and 4 weeks after sciatic nerve ligation, which was defined as "Factor 3", to identify the "neuropathic pain-associated anxiety-related factor". The key finding of the present study, according to the pathway analysis, was that sciatic nerve ligation for 4 weeks, but not 1 week, changed the mRNA expression of ribosomal proteins in the amygdala. Related to this mRNA analysis, in the analysis of miRNA expression, the target genes of miRNAs that were found to be alterd in "Factor 3" included these ribosomal proteins. These results suggest that changes in the expression of ribosomal proteins in the amygdala could lead to pain-associated emotional deficits under a neuropathic pain-like state.

Ribosomal proteins are known to be involved in the basic functions of a cell[17]. Furthermore, the mammalian target of rapamycin (mTOR)-signaling pathway, which can phosphorylate downstream molecules such as p70 ribosomal S6 protein kinase (p70S6K) and then activate S6 ribosomal proteins to initiate new protein synthesis, is one of the most important mechanisms that regulates neuronal excitability and modulates long-term plasticity associated with learning and memory. In addition, p70S6K can be increased in the amygdala of rats with retrieval-extinction manipulation after fear-conditioning[18]. On the other hand, it has been reported that he mTOR-signaling pathway in the brain is activated along with the induction and maintenance of pain-activated hypersensitivity[19]. These findings suggest that persistent neuropathic pain-like stimuli reduce the cell function associated with neuronal plasticity in the amygdala, and in turn, induce emotional deficits including anxiety.

In conclusion, the transomic analysis of changes in gene and protein expression in the amygdala following peripheral nerve injury should be pursued further, and we propose that the change in the expression of ribosomal proteins associated with regulatory miRNA in the amygdala after nerve injury could lead to the pain-associated emotional deficits under a neuropathic pain-like state.

Conflict of interest

The authors have declared no conflicts of interest.

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