Proteomic analysis of rat brains in a model of neuropathic pain following exposure to electroconvulsive stimulation

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ABSTRACT

Some reports have shown that electroconvulsive shock therapy is effective for treating refractory neuropathic pain. However, its mechanism of action remains unknown. This study analyzes changes in protein expression in the brainstems of neuropathic pain model rats with or without electroconvulsive stimulation (ECS). A neuropathic pain model rat is produced by chronic constrictive injury (CCI) of the sciatic nerve. An ECS was administered to rodents once daily for 6 days after the CCI operation. After ECS, the latency to withdrawal from thermal stimulation was significantly increased. The expression of several proteins was changed after CCI. Ten proteins that increased after CCI then had decreased expression levels (close to control) after ECS, and 8 proteins that decreased after CCI then had increased expression levels (close to control) after ECS. In conclusion, ECS improved thermal hypersensitivity in a rat CCI model. Proteomic analysis showed that altered expression levels of proteins in the brainstem of CCI model rats returned to close to control levels after ECS, including many proteins associated with pain. This trend suggests an association of ECS with improved hypersensitivity, and these results may help elucidate the mechanism of this effect.

Electroconvulsive shock therapy (ECT) has been used widely as an effective and established treatment for refractory depression and schizophrenia, although the mechanism of action of this treatment has not been clarified precisely. Many recent studies in the psychiatric field have suggested that the expression levels of the genes for various cerebral neurotransmitters would change after ECT. This change in gene expression might be a possible mechanism of action of ECT (2, 24, 35, 40). Also, some reports have shown that ECT is effective for treating refractory neuropathic pain (5, 16, 22, 36, 37). However, ECT has not been performed as a general treatment for neuropathic pain because the evidence for its therapeutic effect is insufficient, ow-

ing to a lack of data from large-scale controlled trials. Side effects, such as amnesia and injury from ECT, make this option rather unpopular (1). The mechanism of action of ECT in treating neuropathic pain has not been examined sufficiently, and thus remains unclear. We have previously shown that electroconvulsive stimulation (ECS) improves symptoms in rats with neuropathic pain. In the same study we also demonstrated that neuropeptide Y (NPY) expression in the brain was altered (28).

Brainstem tissue was selected for our studies because it contains important nuclei involved in descending inhibition and facilitation of nociceptive or pain transmission, as well as ascending pain transmission and integration with higher structures such as the limbic system. The brainstem nuclei involved in descending control or ascending integration include the rostral ventromedial medulla (RVM), the parabrachial nuclei, the dorsal reticular nuclei and the solitary tract nucleus (25).

We undertook a comprehensive study to clarify

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the mechanism of action of ECT in treating neuropathic pain. To do this, we examined changes in protein expression in the brainstem after ECS in a rat model of neuropathic pain produced by chronic constrictive injury (CCI) of the sciatic nerve. We used proteomic fractionation by 2-dimensional electrophoresis (2-DE) and differential in-gel electrophoresis (DIGE), followed by mass spectrometry (MS) to analyze the protein expression. To our knowledge, this is the first comprehensive report to study the mechanism underlying the effect of ECS on thermal hypersensitivity. The aim was to identify the proteins with altered expression levels in the brainstem after ECS in a rat model of neuropathic pain. Identifying these proteins should be valuable for understanding the mechanism of action of ECT in treating neuropathic pain.

MATERIALS AND METHODS

Production of a neuropathic pain model. The experimental procedures were approved by the institutional committee on Laboratory Animals of the Nippon Medical School (approval number 22-043) and were performed under the guidelines of the International Association for the Study of Pain (9). Male Sprague-Dawley rats (6–7 weeks of age, weighing 200–250g; Saitama Experimental Animals, Saitama, Japan) were used for all experiments. 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 three groups; 1) a group of rats undergoing no procedure (control group, $n=6$); 2) a group of CCI model rats to which ECS was not administered (CCI group, $n=6$); and 3) a group of CCI model rats to which ECS was administered (CE group, $n=6$).

Experimental neuropathy was produced according to a method described in detail elsewhere (4). All surgical procedures were performed on rats that were deeply anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). The left (ipsilateral) common sciatic nerve was exposed in the left mid-thigh and loosely ligated using 4–0 silk thread in four regions, at about 1 mm intervals, to cause CCI. The right (contralateral) sciatic nerve was similarly exposed but not ligated.

Electroconvulsive stimulation. An ECS was administered transauricularly using a metal forceps as previously described (24, 35, 41, 47). ECS was administered to the CE group of rats once daily for 6

days from the 7th to the 12th postoperative day using a pulse generator (57800 ECT Unit; Ugo Basile, Comerio, Italy) (frequency 100 pulses; pulse width 0.5 ms; shock duration 0.8 s; current 50 mA). The shock elicited a full tonic-clonic seizure lasting 20–30 s in all rats. Rats in the CCI group were exposed to the forceps, but without application of current.

Behavioral tests. Two behavioral tests (thermal and mechanical stimulation tests) were performed eight times to assess the pain threshold as previously described (38, 41). These eight times were: the day before surgery (Day 0); 2, 4 and 6 days after the surgery (Day 2, Day 4 and Day 6); 15 min before the second, fourth and sixth ECS (Day 8, Day 10 and Day 12); and 48 h after the last ECS (Day 14) (Fig. 1). The Plantar Test (Model 7370, Ugo Basile) was used to examine thermal hyperalgesia. 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 independently. The latency of paw withdrawal from thermal stimuli was measured three times at 5 min intervals and the average value was used as the latency of the response. Mechanical allodynia was measured using a set of von Frey filaments (Touch-test sensory evaluator; Muromachi Kikai, Saitama, Japan) with bending forces ranging from 1.0 to 23.0 g. Each rat was placed on a metallic mesh floor, covered with a plastic box, and a von Frey filament was applied from under the mesh floor to the plantar surface of either the contralateral or ipsilateral hindpaw. Each paw was stimulated

Fig. 1 Experimental protocol. Neuropathic pain was produced by chronic constrictive injury (CCI) of the sciatic nerve. Using a pulse generator, an electroconvulsive shock (ECS) was administered to rodents once daily for 6 days on days 7–12 after the CCI operation. Thermal and mechanical stimulation tests were performed to assess pain thresholds on the day before surgery (Day 0); 2, 4 and 6 days after the surgery (Day 2, Day 4 and Day 6), 15 min before the second and fourth and sixth ECS (Day 8, Day 10 and Day 12), and 48 h after the last stimulation, the rats were decapitated 48 h after the last stimulation, the brains were rapidly removed and the brainstems were separated.

with each filament five times at 10 second intervals in the individual trials. The weakest force (g) inducing withdrawal of the stimulated paw at least three times in each trial was referred to as the paw withdrawal threshold. The difference in latency values for the two sides (difference score) was calculated as follows: difference score = latency on the contralateral side – latency on the ipsilateral side.

Differential in-gel electrophoresis. Rats were decapitated 48 h after the last stimulation. The brains were rapidly removed and the brainstems were separated as previously described (3). The cerebellum of the hindbrains was removed. The brainstem was defined as the area from the caudal medulla (cranial to cervical 1 spinal cord) to the area parallel to the cranial aspect of the cerebellum just caudal to the midbrain and at the most cranial aspect of the third ventricle. Brainstems were immediately frozen in liquid nitrogen and stored at -80°C .

As described above, three groups of rats were examined. For each of the three groups, a total of 18 two-dimensional gels were produced for DIGE analysis. Each gel contained three different fluorescently labeled samples as previously described (32). An equal amount of protein homogenate from each of the three groups was pooled and labeled with the Cy2 (cyanine 2) dye. This served as the internal standard used in all 18 gels. Aliquots of the six controls were pooled and labeled with Cy3 (cyanine 3) dye to serve as the internal control sample. Finally, samples of all groups were labeled individually with Cy5 (cyanine 5) dye.

Sample preparation and labeling. For each individual brainstem tissue sample, lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS) and 1 mM protease inhibitors (GE Healthcare Amersham Biosciences, Uppsala, Sweden) were added. The tissue was homogenized and centrifuged at $12,000 \times g$ for 15 min to remove nuclei and undissolved materials. The supernatants were centrifuged for 1 h at $10,000 \times g$ and then collected. The protein homogenate was quantitated at 595 nm using the Bradford method (BioRad, Hercules, CA, USA). The Cy2, Cy3, and Cy5 labeling followed standard methods for minimal labeling. Briefly, the powdered Cy2, Cy3, or Cy5 dyes were dissolved in dimethylformamide (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 1 mM. An aliquot of 50 μg protein from each sample (pooled or individual) was labeled with 400 pmol of dye. Labeling was performed for 30 min on ice in the dark, before the reactions were

quenched by adding 10 mM lysine (1 mL for each 400 pmol of dye) for 10 min on ice in the dark. The quenched Cy2, Cy3, and Cy5 labeled samples (each containing 50 μg protein) were then pooled and mixed with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% DTT (Sigma-Aldrich) to a final volume of 450 μL .

2-Dimensional electrophoresis. For the first dimension, isoelectric focusing (IEF) gradient strip gels were used. The 450 μL samples were passively rehydrated into 24 cm pH 4–7 IPG strips (GE Healthcare Amersham Biosciences) for 10 h, followed by IEF using an IPGphor IEF unit (GE Healthcare Amersham Biosciences) for a total of 14800 Vh. The second-dimension, SDS-PAGE, was performed on 20 cm $\times$ 24 cm 12.5% polyacrylamide gels using low-fluorescence glass plates. Electrophoresis was carried out using an Ettan DALTsix system (GE Healthcare Amersham Biosciences) at 6 W 80 V for 1 h followed by 12 W 150 V for 14 h in the dark. Gels were scanned immediately using the same setting and scanner in all cases.

Imaging and data processing. The differentially labeled DIGE gels were imaged at a resolution of 100 μm separately by dye-specific excitation and emission wavelengths using a Typhoon 9400 Variable Mode Imager (GE Healthcare Amersham Biosciences). The gel intensities were kept in the linear range of 60,000–90,000 pixels/gel for image capture. Tagged image file format images were cropped and exported for analysis using the DeCyder v5.0 suite of software tools (GE Healthcare Amersham Biosciences). The differential in-gel analysis (DIA) module was used to identify protein spots and quantitatively compare the expression ratio of each individual protein spot-feature from Cy5 to the Cy3 labeled sample on a given gel after normalization to the Cy2 signal. This in-gel normalization reduces the requirement for technical repeats and enhances quantitative sensitivity (23). Spots are matched across gels by a combination of automated detection and manual examination. Briefly, a group of 200–250 spots in each gel of the entire group of 18 gels were matched to those in an arbitrary master gel by eye using the common Cy2 channel. These manually matched spots were evenly distributed across the gel and then served as landmarks for the DIGE DeCyder software to automatically match the rest of the spots on each gel. Matches were confirmed manually, and the processes were repeated iteratively. The DIA datasets for each individual gel were then

collectively analyzed using the biological variation analysis (BVA) module, which allowed for matching across gels using the common Cy2 channel. This allows for reporting the volume of Cy2, Cy3, and Cy5 channels in all gels (spot volume = spot intensity/gel intensity) and for comparing protein migration patterns. Images and intensity data were collected as text limited files.

Mass spectroscopic identification. Two-dimensional gels were stained with Deep Purple Total Protein Stain (GE Healthcare Amersham Biosciences). In this procedure, gels were fixed 1 h after electrophoresis in 7.5% acetic acid and 10% methanol at room temperature. Gels were washed with 35 mM sodium hydrogen carbonate and 300 mM sodium carbonate at room temperature for 30 min with gentle agitation and then stained with Deep Purple Total Protein Stain for 1 h at room temperature in the dark. Gels were washed twice in 7.5% acetic acid for 15 min at room temperature in the dark.

Proteins of interest were robotically excised, equilibrated with 100 mM ammonium bicarbonate for 20 min and dehydrated with two 10 min incubations with 100% acetonitrile in a 96-well plate format using Ettan Spot Picker and Digester workstations (GE Healthcare Amersham Biosciences). Dehydrated gel plugs were manually digested in-gel with 25 mM sequencing grade modified trypsin protease (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate for 16 h at 37°C. Tryptic peptides were then extracted from the gel plugs in two cycles of 1% trifluoroacetic acid using the Ettan Digester workstation and dried by vacuum centrifugation. Peptides were reconstituted in 20 μ L 0.1% trifluoroacetic acid.

The resulting digested proteins were subjected to a high-pressure liquid chromatography separation on the chromatography system (MAGIC 2002; Michrom BioResources, Auburn, CA, USA). This chromatography system was coupled via a HTS-PAL (CTC Analytics, Zwingen, Switzerland) to an ion trap MS (LCQ DECA XP; Thermo Finnigan, CA, USA). The resulting MS and MS/MS spectra of peptide ions were searched using MASCOT software (www.matrix-science.com). The peptide species count is the number of peptides used for the MASCOT scoring report. Any MASCOT score > 70 is considered significant.

Statistical analysis. The latencies and threshold values in behavioral tests are expressed as means $\pm$ standard deviation (SD). A paired *t*-test compared

those values between the ipsilateral and contralateral sides. An unpaired *t*-test compared the difference score between the CE and CCI groups. Dunnett's test for multiple comparisons compared latencies, threshold values, or difference scores obtained in behavioral tests performed before the operations with those obtained in tests performed after the operations. $P < 0.05$ is considered to be statistically significant. Also, CyDye-stained gels were digitized and the images were analyzed using DeCyder 2D software (GE Healthcare Amersham Biosciences). A one-way ANOVA (analysis of variance) followed by a Student-Newman-Keuls post hoc test was used to test for differences between the control and CCI groups, and between the CCI and CE groups. All results were reported as means $\pm$ SD, and $P < 0.01$ was considered statistically significant.

RESULTS

Behavioral tests

Compared with the values on Day 0, latencies of paw withdrawal from thermal stimulation (Figs. 2A, 2B) and paw withdrawal thresholds in response to mechanical stimulation on the ipsilateral side (Figs. 3A, 3B) were decreased on Day 2, 4, 6, 8, 10 and 12. After ECS, the latencies of paw withdrawal from thermal stimulation on the ipsilateral side were increased on Day 14 in the CE group (Fig. 2A) and the difference score was significantly decreased on day 14 (Fig. 2C). Paw withdrawal thresholds in response to mechanical stimulation were not significantly changed after ECS (Fig. 3A). The results show that ECS is effective for treating thermal hypersensitivity, but not for treating mechanical allodynia.

Protein fraction and 2-dimensional map

In the scanned gels from the brainstem region, both volume and area could be determined for approximately 2500 spots. To establish a significant area of overlapping between the maps, a subset of 1400 representative spots was chosen.

There were 32 proteins in the CCI group with significantly increased expression compared with the control group, and each protein was up-regulated with an approximately 1.2–1.4 fold change (Table 1). Based on their physiologic function, these regulated proteins can be roughly subdivided into fundamental categories, such as: proteins related to cellular homeostasis and metabolism; neuronal function proteins; heat shock proteins, chaperones, and antioxidants; proteins related to cell cycle, apoptosis,

Thermal hypersensitivity

Fig. 2 Effects of electroconvulsive shock (ECS) administered to chronic constrictive injury (CCI) model rats (A) ($n = 6$) and the effects of CCI procedure (B) ($n = 6$) 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 CCI rats are shown. Difference scores were calculated as the value of the withdrawal latency on the ipsilateral side subtracted from the latency on the contralateral side (C) (CE group, $n = 6$; CCI group, $n = 6$). The degree of reduction of thermal hyperalgesia by ECS is statistically significant (Day 14). * $P < 0.05$, compared with values on the contralateral side on the same days by a paired t -test; $^{\dagger}P < 0.05$, compared with the CCI group by an unpaired t -test; $^{\#}P < 0.05$, compared with the values on the preoperative day (Day 0) by Dunnett's multiple comparisons. Values are means $\pm$ standard deviation.

and neurodegeneration; or proteins related to the immune system. Among these proteins, 10 proteins had significantly decreased expression in the CE group compared with the CCI group, with expression levels reduced to close to control levels (approximately 0.9–1.2 fold change compared with the control group). The name, function and expression ratio of each protein is tabulated in Table 2. RAP1 (GTP-GDP dissociation stimulator 1), CNDP dipeptidase 2, ATP synthase beta subunit, phosphoglycerate kinase (PGK), 3'-5'-bisphosphate nucleotidase 1, Ppa1 protein, and inositol monophosphatase 1 were reduced to control levels. Dihydrolipoamide S-acetyltransferase, malate dehydrogenase 1, and the subunit d of mitochondrial H^+ ATP synthase were

reduced to close to control levels.

There were 17 proteins in the CCI group with significantly decreased expression compared with the control group, and each protein was down-regulated with approximately 0.5–0.8 fold change (Table 3). Among these proteins, 8 proteins had significantly increased expression in the CE group compared with the CCI group, with expression levels increased to close to control levels (approximately 0.7–1.1 fold change compared with the control group). The name, function and expression ratio of each protein is tabulated in Table 4. Collapsin response mediator protein, rCRMP-4, alpha-internexin and glial fibrillary acidic protein (GFAP) were increased to control levels. Heat shock protein 4,

Mechanical allodynia

Fig. 3 Effects of electroconvulsive shock (ECS) administered to chronic constrictive injury (CCI) model rats (A) ($n = 6$) and the effects of the CCI procedure (B) ($n = 6$) 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 CCI rats are shown. Difference scores were calculated as the value of the withdrawal threshold on the ipsilateral side subtracted from the threshold on the contralateral side (C) (CE group, $n = 6$; CCI group, $n = 6$). ECS was not effective for treating mechanical allodynia. * $P < 0.05$ compared with values on the contralateral side on the same days, by a paired t -test; # $P < 0.05$ compared with the values on the preoperative day (Day 0) by Dunnett's multiple comparisons. Values are means $\pm$ standard deviation.

grp75, aminopeptidase-B and the cytosolic branch chain aminotransferase (BCATc) were increased to close to control levels.

Eighteen proteins that were modulated following CCI returned to control levels after ECS. Images and Spot ID of these proteins were shown (Fig. 4).

DISCUSSION

In the present study, ECS is effective for treating thermal hypersensitivity, as has been reported in a previous study (41). The expression of several proteins in the brainstem was changed after CCI, and ECS regulated 18 proteins that changed after CCI to control expression level. In the following para-

graphs, we will describe the relationship between these proteins and the mechanism of treating neuropathic pain.

CNDP dipeptidase 2 is a carnosine splitting enzyme that occurs in the brain and controls the level of carnosine (29). Carnosine has both antioxidant and neuroprotective effects against oxidative stress (34). Oxidative stress in the brain caused by CCI may increase the expression of CNDP dipeptidase 2 (1.34 ± 0.09 compared to the control group). After ECS, the expression of this protein was reduced to the control level (1.09 ± 0.03 compared to the control group). This suggests that the expression level can be modified by ECS, and the alteration of CNDP dipeptidase 2 might correlate with a mecha-

Table 1 Proteins in the CCI group with significantly increased expression compared with the control group

Protein identification						Expression ratio
Spot ID	Protein score mascot	Accession No	Mw (kDa) / pI	Sequence Coverage (%)	Protein	CCI
Proteins related to cellular homeostasis and metabolism						
820	107	NM053748	83.3 / 5.12	8	dipeptidyl peptidase III	1.18 ± 0.05
1024	831	NM130428	72.5 / 6.75	26	succinate dehydrogenase complex subunit A	1.25 ± 0.06
1044	555	NM031025	67.6 / 8.76	19	dihydrolipoamide S-acetyltransferase	1.28 ± 0.04
1387	254	AY034137	48.6 / 6.06	14	aldehyde dehydrogenase	1.27 ± 0.16
1400	284	NM001025063	46.9 / 4.73	18	secernin 1	1.22 ± 0.06
1421	544	M19044	51.1 / 4.92	27	ATP synthase beta subunit	1.24 ± 0.12
1553	559	NM001004250	53.5 / 5.57	27	ubiquinol-cytochrome c reductase core protein I precursor	1.36 ± 0.10
1689	104	M31788	44.9 / 7.53	7	phosphoglycerate kinase (PGK)	1.66 ± 0.08
1692	165	NM001005383	46.1 / 5.95	12	aminoacylase 1 (ACY1)	1.27 ± 0.03
1711	569	BC166998	47.0 / 6.06	35	Sucla2 protein	1.15 ± 0.05
1814	146	NM171990	33.4 / 5.58	12	3'-5'-bisphosphate nucleotidase 1	1.33 ± 0.18
1940	278	NM053638	40.0 / 6.47	17	isocitrate dehydrogenase 3 alpha precursor	1.15 ± 0.07
2007	553	NM012595	36.8 / 5.7	41	lactate dehydrogenase B (LDH B)	1.16 ± 0.13
2085	526	NM033235	36.6 / 6.16	34	malate dehydrogenase 1	1.24 ± 0.30
2100	238	BC099794	38.2 / 6.42	21	Ppa1 protein	1.19 ± 0.05
2166	235	NM080585	33.6 / 5.3	21	N-ethylmaleimide-sensitive factor attachment protein alpha	1.25 ± 0.17
2485	249	D10021	18.8 / 5.78	45	subunit d of mitochondrial H ⁺ ATP synthase	1.31 ± 0.09
Neuronal function proteins						
1295	364	NM001107728	52.6 / 5.53	21	RAP1 (GTP-GDP dissociation stimulator 1)	1.24 ± 0.12
1377	193	NM001010920	53.1 / 5.43	15	CNDP dipeptidase 2	1.34 ± 0.09
2246	249	NM032057	30.8 / 5.17	30	inositol monophosphatase 1	1.16 ± 0.04
Heat shock proteins, chaperones, and antioxidant proteins						
816	779	NM001005550	80.3 / 5.65	28	NADH dehydrogenase Fe-S protein 1	1.17 ± 0.07
1150	650	CH473989	60.1 / 5.38	30	chaperonin subunit 8 isoform CRA	1.39 ± 0.11
1155	565	NM001004078	59.9 / 5.51	24	chaperonin containing TCP 1 subunit 5	1.23 ± 0.04
1225	449	M26653	55.3 / 6.19	21	glucose-6-phosphate dehydrogenase	1.32 ± 0.17
1326	594	NM001005905	57.7 / 6.01	26	chaperonin containing TCP 1	1.19 ± 0.08
1429	466	CH473991	49.4 / 6.23	25	rCG53214 isoform CRA	1.46 ± 0.20
1526	775	BC090069	47.4 / 6.16	47	enolase 1	1.44 ± 0.02
1548	379	CH473964	34.9 / 4.87	22	enolase 2 gamma isoform CRA	1.24 ± 0.12
1559	753	NM139325	47.5 / 5.03	44	enolase 2	1.37 ± 0.05
1923	204	NM199495	40.7 / 7.64	12	NADH dehydrogenase 1 alpha subcomplex 10 precursor	1.47 ± 0.08
1980	254	NM022297	31.8 / 5.75	15	dimethylarginine dimethylaminohydrolase 1 (DDAH1)	1.21 ± 0.10
Proteins related to the immune system						
869	314				glycyl-tRNA synthetase	1.37 ± 0.16

n = 6 in each group. Values are mean ± standard deviation. All proteins listed above are significantly increased compared with the control group ($P < 0.01$).

Expression ratio: Relative expression level of the protein in the CCI group of rats compared with the control group.

CCI: chronic constrictive injury. MW: molecular weight. pI: isoelectric point.

nism for treating neuropathic pain.

Inositol monophosphatase 1 is associated with phosphatidyl inositol metabolism. Its activation of the metabolic pathway mediated by serotonin via the 5HT₂ receptor has an antidepressive effect (11, 31, 33). Serotonin (5HT) is part of the descending inhibitory system in the central nervous system, but 5HT might be inhibitory or facilitatory (30, 42, 44,

49). The increased expression level of inositol monophosphatase 1 in the brainstem after CCI (1.16 ± 0.04 compared to the control group) and the return to the control level after ECS (1.03 ± 0.08 compared to the control group) suggest that ECS has an effect on the descending inhibitory system and improves neuropathic pain.

ATP synthase beta subunit and subunit d of mito-

Table 2 Proteins with significantly decreased expression in the CE group compared with the CCI group

Spot ID	Protein identification		Expression ratio	
	Protein	Function	CCI	CE
1044	dihydrolipoamide S-acetyltransferase	Part of the mitochondrial pyruvate dehydrogenase complex	1.28 ± 0.04*	1.13 ± 0.03*
1295	RAP1 (GTP-GDP dissociation stimulator 1)	GTP-binding protein involved in the control of cell-cell and cell-matrix adhesion	1.24 ± 0.12*	0.97 ± 0.09
1377	CNDP dipeptidase 2	Neuroprotection protein	1.34 ± 0.09*	1.09 ± 0.03
1421	ATP synthase beta subunit	ATP production	1.24 ± 0.12*	1.05 ± 0.07
1689	phosphoglycerate kinase (PGK)	A transferase enzyme used in the seventh step of glycolysis	1.66 ± 0.08*	0.95 ± 0.17
1814	3'-5'-bisphosphate nucleotidase 1	Protein of converting adenosine 3'-phosphate 5'-phosphosulfate (PAPS) to adenosine 5'-phosphosulfate (APS) and 3'-phosphoadenosine 5'-phosphate (PAP)	1.33 ± 0.18*	1.07 ± 0.06
2085	malate dehydrogenase 1	A pivotal enzyme in the malate aspartate shuttle	1.24 ± 0.30*	1.16 ± 0.04*
2100	Ppa 1 protein	A mechanism favoring biosynthesis	1.19 ± 0.05*	0.95 ± 0.15
2246	inositol monophosphatase 1	Key enzyme of the phosphatidyl inositol signaling pathway	1.16 ± 0.04*	1.03 ± 0.08
2485	subunit d of mitochondrial H ⁺ ATP synthase	ATP production	1.31 ± 0.09*	1.22 ± 0.04*

n = 6 in each group. Values are mean ± standard deviation. All proteins listed above are significantly decreased in the CE group compared with the CCI group ($P < 0.01$). Proteins of significantly changed compared with control group ($*P < 0.01$).

Expression ratio (CCI): Relative expression level of the protein in the CCI group of rats compared with the control group.

Expression ratio (CE): Relative expression level of the protein in the CE group compared with the control group.

CE: chronic constrictive injury (CCI) model rats to which an electroconvulsive shock (ECS) was administered.

chondrial H⁺ ATP synthase are involved in ATP production. ATP is a significant intercellular messenger molecule that interacts with purinoceptors. Specifically, the P2X and P2Y receptors, which are localized in the central and peripheral nervous systems, play an important role in excitatory nociceptive processing (6, 21). The results of the present study showed that ATP synthase expression is increased by chronic pain (ATP synthase beta subunit, 1.24 ± 0.12 compared to the control group; subunit d of mitochondrial H⁺ ATP synthase, 1.31 ± 0.09 compared to the control group), but that it is restored to the same level as seen in the control group after ECS (ATP synthase beta subunit, 1.05 ± 0.07 compared to the control group; subunit d of mitochondrial H⁺ ATP synthase, 1.22 ± 0.04 compared to the control group). This suggests a correlation between ATP synthase levels and the effects of ECS on chronic pain.

Other proteins that demonstrated increased expression after CCI include RAP1/GTP-GDP dissociation stimulator 1 (related to neuronal function), phosphoglycerate kinase (PGK), 3'-5'-bisphosphate nucleotidase 1, malate dehydrogenase 1, and Ppa 1 protein (related to cellular homeostasis and metabolism). These proteins were reduced to control levels after ECS. However, their physiological roles to

thermal hypersensitivity are unclear at this time.

Also we noted that some proteins for which expression decreased after CCI had increased expression levels (close to control levels) after ECS. The subsequent paragraphs describe these proteins.

HSP₄ and grp75 are heat shock proteins that have increased expression and show a nerve-protective action in inflammation and various disorders (8, 15, 19). Increased expression of these proteins has been found in the dorsal root ganglion (DRG) after nerve injury (17, 18, 46, 48). Expression of HSP₄ and grp75 in the brainstem has not been reported previously, but in the present study their expression decreased in the brainstem after CCI (HSP₄, 0.75 ± 0.07 compared to the control group; grp75, 0.65 ± 0.05 compared to the control group). The return of the expression levels of these proteins to close to control levels after ECS (HSP₄, 0.86 ± 0.03 compared to the control group; grp75, 0.72 ± 0.05 compared to the control group) suggests that ECS can restore the neuroprotection provided by HSP₄ and grp75.

Aminopeptidase-B is involved in the production of neuropeptide Y (NPY) (12). NPY is a neuropeptide that is widespread throughout the central nervous system (CNS), and studies show NPY could be related to pain (10, 13, 45). Taylor *et al.* showed

Table 3 Proteins in the CCI group with significantly decreased expression compared with the control group

Protein identification						Expression ratio
Spot ID	Protein score mascot	Accession No	Mw (kDa) / pI	Sequence Coverage (%)	Protein	CCI
<i>Proteins related to cellular homeostasis and metabolism</i>						
1040	193	D10655	57.6 / 5.53	13	dihydrolipoamide acetyltransferase	0.83 ± 0.11
1338	267	NM057213	56.8 / 5.57	11	vacuolar H ⁺ ATPase B2	0.72 ± 0.05
1393	497	M19044	51.1 / 4.92	30	ATP synthase beta subunit	0.79 ± 0.05
1642	488	CH474034	36.1 / 6.27	42	creatine kinase brain isoform CRA a	0.72 ± 0.08
1743	83	M18668	42.9 / 5.33	5	creatine kinase	0.55 ± 0.05
<i>Neuronal function proteins</i>						
999	113	D87515	73.2 / 5.47	8	aminopeptidase-B	0.57 ± 0.13
1397	424	U03700	49.9 / 5.35	26	glial fibrillary acidic protein (GFAP)	0.57 ± 0.20
1646	88	AF165887	41.2 / 7.2	5	cytosolic branch chain aminotransferase (BCATc)	0.76 ± 0.10
<i>Heat shock proteins, chaperones, and antioxidant proteins</i>						
558	280	NM001106428	55.0 / 5.5	8	heat shock protein 4	0.75 ± 0.07
905	425	S78556	73.9 / 5.87	16	grp75	0.65 ± 0.05
940	1069	M19141	71.0 / 5.37	34	heat shock protein 70	0.70 ± 0.07
997	348	CH479375	48.6 / 6.16	22	rCG57965 isoform CRA b	0.77 ± 0.09
1541	306	NM139125	47.5 / 5.03	16	enolase 2	0.70 ± 0.14
<i>Proteins related to cell cycle, apoptosis, and neurodegeneration</i>						
1015	93	U52095	62.5 / 6.61	5	collapsin response mediator protein	0.75 ± 0.17
1109	75	U52104	39.3 / 5.08	7	rCRMP-4	0.50 ± 0.20
1126	403	X52017	55.7 / 5.2	23	alpha-internexin	0.73 ± 0.09
2161	150	AF051895	33.9 / 4.99	12	lipocortin V	0.84 ± 0.12

n = 6 in each group. Values are mean ± standard deviation. All proteins listed above are significantly decreased compared with the control group ($P < 0.01$).

Expression ratio: Relative expression level of the protein in the CCI group of rats compared with the control group.

CCI: chronic constrictive injury. MW: molecular weight. pI: isoelectric point.

Table 4 Proteins with significantly increased expression in the CE group compared with the CCI group

Spot ID	Protein	Protein identification		Expression ratio	
		Function		CCI	CE
558	heat shock protein 4	Neuroprotection after a variety of stresses or injuries		0.75 ± 0.07*	0.86 ± 0.03*
905	grp75	Neuroprotection after a variety of stresses or injuries		0.65 ± 0.05*	0.72 ± 0.05*
999	aminopeptidase-B	Production of neuropeptide Y (NPY)		0.57 ± 0.13*	0.70 ± 0.08*
1015	collapsin response mediator protein	A member of a family of proteins that are involved in axonal growth		0.75 ± 0.17*	1.11 ± 0.18
1109	rCRMP-4	A member of a family of proteins that are involved in axonal growth		0.50 ± 0.20*	1.16 ± 0.08
1126	alpha-internexin	A neuronal intermediate filament protein implicated in neurodegenerative disease		0.73 ± 0.09*	1.03 ± 0.13
1397	glial fibrillary acidic protein (GFAP)	An intermediate filament protein found in the cytoskeleton of astroglia		0.57 ± 0.20*	0.85 ± 0.23
1646	cytosolic branch chain aminotransferase (BCATc)	Brain derived neurotrophic factor (BDNF) that are believed to have several actions in the CNS		0.76 ± 0.10*	0.85 ± 0.05*

n = 6 in each group. Values are mean ± standard deviation. All proteins listed above are significantly increased in the CE group compared with the CCI group ($P < 0.01$). Proteins of significantly changed compared with control group ($*P < 0.01$).

Expression ratio (CCI): Relative expression level of the protein in the CCI group of rats compared with the control group.

Expression ratio (CE): Relative expression level of the protein in the CE group compared with the control group.

CE: chronic constrictive injury (CCI) model rats to which an electroconvulsive shock (ECS) was administered.

Fig. 4 A typical 2-dimensional map. Spots were marked by their spot ID numbers shown in Table 2 and Table 4.

that neuropathic pain was improved by administration of NPY to the RVM, and proposed that NPY would have an inhibitory action on pain (45). In a previous study performed in our facility using CCI model rats, the expression level of NPY decreased after CCI, but returned to the control level after ECS (28). In the present study, the trend of decreased expression after CCI (0.57 ± 0.13 compared to the control group) and a return to close to the control level after ECS (0.70 ± 0.08 compared to the control group) was found again. This correlates to an effect of ECS on chronic pain and NPY levels.

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein of astrocytes, but whether changes in expression of this protein contribute to pain processing is not known. Expression of GFAP is increased in the periaqueductal gray matter and DRG in neuropathic pain model rats (14, 27, 43). Although the decreased expression level (0.57 ± 0.20 compared to the control group) of GFAP in the brainstem after CCI shows a different trend to that previously reported, the return to the control level (0.85 ± 0.23 compared to the control group) after ECS suggests that ECS has an effect on astrocytes which improve hypersensitivity.

Cytosolic branch chain aminotransferase (BCATc) is associated with brain derived neurotrophic factors (BDNFs) that are believed to have several actions in the CNS (7). The decreased expression level of BCATc after CCI (0.76 ± 0.10 compared to the control group) was increased to close to the control level after ECS (0.85 ± 0.05 compared to the control group). This suggests a correlation between BCATc and the effects of ECS on chronic pain.

Collapsin response mediator proteins might be involved with axonal growth in the CNS (39). Alpha-internexin is a neuronal intermediate filament protein in the CNS that is involved in neurodegenerative diseases (20). These proteins that related to cell cycle, apoptosis, and neurodegeneration were altered greatly, and have a broad impact on the CNS, which may include involvement in hypersensitivity to pain.

Previously, a number of proteomic studies have been performed using different types of neuropathic pain models, and some proteins related to neuropathic pain have already been reported (26). In our study, we demonstrated that ECS improved thermal hypersensitivity, and 18 proteins that were modulated following CCI returned to control levels after

ECS. These 18 proteins are involved in neuronal degeneration, regeneration and inflammatory processes like previous reports. Although we could not clearly make the interaction of those proteins mentioned above from our results, our findings strongly suggest that ECS affects neuropathic pain and expression of some proteins related to neuropathic pain. Further studies need to be conducted to reveal the correlation between thermal hypersensitivity and each individual protein.

In conclusion, ECS improved thermal hypersensitivity in a CCI rat model. The expression levels of eighteen proteins that were modulated following CCI, including CNDP dipeptidase 2, inositol monophosphatase 1, ATP synthase beta subunit, subunit d of mitochondrial H⁺ ATP synthase, HSP₄, grp75, aminopeptidase-B, GFAP and BCATc, returned to control levels after ECS. We expect that this trend is the key to understanding the mechanism of action of ECT in treating neuropathic pain.

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