Original Research Article

Electroconvulsive Stimulation (ECS) Increases the Expression of Neuropeptide Y (NPY) in Rat Brains in a Model of Neuropathic Pain: A Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Study

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ABSTRACT

Objectives. Electroconvulsive shock therapy (ECT) has been widely used as an effective and established treatment for refractory depression and schizophrenia. Some reports have shown that ECT is also effective for treating refractory neuropathic pain.

Design. In a rat model of neuropathic pain produced by chronic constrictive injury (CCI) of the sciatic nerve, thermal hyperalgesia, and mechanical allodynia were observed from day 2 after surgery. An electroconvulsive shock (ECS) was administered to rodents once daily for 6 days on days 7–12 after CCI operation using a pulse generator. Thermal and mechanical stimulation tests were performed to assess pain thresholds. Real-time polymerase chain reaction was used to measure the gene expression levels for 5HT1AR, 5HT2AR, neuropeptide Y (NPY), and GABAaR in the brain.

Results. After ECS, the latency to withdrawal from thermal stimulation was significantly increased; however, pain withdrawal thresholds in response to mechanical stimulation were not significantly changed. Expression ratios of NPY were significantly greater after ECS.

Conclusion. Symptoms of neuropathic pain improved and expression of NPY in the brain was increased in CCI model rats after ECS, suggesting that changes in the expression of NPY in the brain may be related to the mechanism of action of ECT in treating neuropathic pain.

Key Words. ECS; ECT; CCI; Neuropathic Pain; NPY; RT-PCR

Introduction

Electroconvulsive shock therapy (ECT) has been widely used as an effective and established treatment for refractory depression and schizophrenia, although the mechanism of action of this treatment has not been precisely clarified. Many recent studies in the psychiatric field have suggested that the levels of expression of the genes for various cerebral neurotransmitters would change after ECT, and this is attracting attention as a possible mechanism of action of ECT [1–4]. On the other hand, some reports have shown that ECT is also effective for treating refractory neuropathic pain [5–9]. However, ECT has not been performed as a general treatment because...
the evidence for its therapeutic effect is insufficient, owing to a lack of data from large-scale controlled trials. Side effects, such as amnesia and injury from ECT, make the option rather unpopular [10].

The mechanism of action of ECT in treating neuropathic pain has not been examined sufficiently, and thus remains unclear. In this study, we tested the hypothesis that ECT might change the gene expression of neurotransmitters related to the descending inhibitory system in the brain, and thereby improve the symptoms of neuropathic pain.

Methods

Experimental Animals
The experimental procedures were approved by the institutional Committee on Laboratory Animals of Nippon Medical School (approval number 19-091) and were performed under the guidelines of the International Association for the Study of Pain [11].

Male Sprague-Dawley rats (6–7 weeks of age and 200–250 g in weight; Saitama Experimental Animals) were used for all experiments. Rats were housed in clear plastic cages with sawdust bedding at standard room temperature, under a 12-hour light/dark cycle. All rats received food and water ad libitum. We divided the rats into three groups: 1) a group of chronic constrictive injury (CCI) model rats to which an electroconvulsive shock (ECS) was administered (ECS group, N = 9); 2) a group of CCI model rats to which ECS was not administered (CCI group, N = 27); and 3) a group of rats undergoing no procedure (control group, N = 9).

Production of a Neuropathic Pain Model
Experimental neuropathy was produced according to a method described in detail elsewhere [12]. 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 as a control.

Electroconvulsive Stimulation
An ECS was administered transauricularly using metal forceps as previously described [2,4,13,14]. ECS was administered to the ECS group of rats (N = 9) once daily for 6 days from the 7th to the 12th postoperative day (days 7–12) using a pulse generator (57800 ECT Unit; Ugo Basile, Comero, 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 seconds in all rats. Rats in the CCI group (N = 9) were exposed to the forceps, but without application of current once daily from the 7th to the 12th postoperative day (days 7–12).

Behavioral Tests
Two behavioral tests (thermal and mechanical stimulation tests) were performed eight times to assess pain thresholds as previously described [14,15]: the day before surgery (day 0), and days 2, 4, 6, 8, 10, 12, and 14 days after the surgery. During the period of ECS, the behavioral tests were performed prior to ECS (days 8, 10, and 12) and 48 hours after the last ECS (day 14). The plantar test (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-minute intervals, and the average value was used as the latency of the response. Mechanical allodynia was measured using a set of von Frey filaments (Muromachi Kikai, Saitama, Japan) with bending forces ranging from 2.0 to 32.0 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 with each filament five times at 10-second intervals in 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.

Real-Time Reverse Transcriptase Polymerase Chain Reaction
Real-time polymerase chain reaction (RT-PCR) was used to measure the levels of gene expression for serotonin 1A receptor (5HT1AR), serotonin 2A receptor (5HT2AR), neuropeptide Y (NPY), and gamma-aminobutyric acid A α1 receptor (GABAAα1R) in whole brains of rats in each group.
Rats were decapitated, and the brains were rapidly removed, frozen in liquid nitrogen, and stored at -80°C. Total mRNA was isolated from each brain using the acid guanidinium phenol chloroform method with Isogen (Wako, Tokyo, Japan). A spectrophotometer was used to determine the quantity of mRNA. Total RNA (8 µg) was used as a template for reverse transcription reactions with 8 µL of 5 × RT buffer, 6 µL of 1.25 mM dNTP mixture, 2 µL of random primer (hexa-deoxyribonucleotide mixture), 2 µL of 0.1 M DTT, 1 µL of Moloney Murine Leukemia Virus Reverse Transcriptase, and 0.2 µL of ribonuclease inhibitor (Applied Biosystems, Foster City, CA). The reverse transcription reaction was carried out using PCR Express (Thermo Fisher Scientific, Waltham, MA) 42°C for 60 minutes, and 4°C for 5 minutes.

The amplification of 5HT1AR, 5HT2AR, NPY, and GABAA1R was performed in a Fast 96-well reaction plate (Applied Biosystems), as previously described [16]. Reactions were carried out in a 20-µL volume containing 10 µL of Taqman Universal PCR master mix, 1 µL of Taqman Gene Expression Assays (Applied Biosystems), 8 µL of RNase-free water (Wako), and 20 ng of cDNA. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to assess DNA integrity. Assay identifications are shown in Table 1.

PCR consisted of an initial denaturation at 95°C for 3 seconds, incubation at 60°C for 30 seconds, and then, measurement of the fluorescence signal after each cycle. The Taqman probe labeled with FAM was cleaved during amplification, generating a fluorescent signal. The assay used an instrument capable of measuring fluorescence in real time (ABI PRISM 7500 Fast Sequence Detector; Applied Biosystems).

RT-PCR data are represented as the threshold cycle (Ct) values, where Ct represents a unitless value defined as the fractional cycle number at which the sample fluorescence signal passes a fixed threshold above baseline. Triplicate samples with markedly different values, which were obviously due to inaccurate operation, were omitted. Relative amounts of all mRNAs were calculated using the comparative Ct method (Applied Biosystems).

ΔCt is the difference in the Ct values derived from the experimental samples and the GAPDH control, and ΔΔCt represents the difference between paired samples, as calculated by the formula: ΔΔCt = ΔCt of sample of CCI + ECS group − ΔCt of sample of CCI + sham-ECS group. The expression ratio shows the relative quantity of the target gene (Xtarget) to the control gene (Xcontrol). The expression ratio was computed using the formula: Xtarget/Xcontrol = 2−ΔΔCt.

**Immunohistochemistry**

We examined an immunohistochemical stainability for NPY in brain sections from rats in each group (control group: N = 3, ECS group: N = 3, CCI group: N = 3) at 14 days after the surgery (day 14). Rats were anesthetized with 50 mg/kg pentobarbital sodium and perfused through the left ventricle with 0.1 M phosphate buffered saline (PBS), followed by a mixture of 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB). Brains were then removed from skulls and postfixed in the same fixative overnight at 4°C, and then soaked in 30% sucrose in 0.1 M PB for 3 days at 4°C for cryoprotection. The brains were quickly frozen using powdered dry ice and cut into 25-µm sections using a cryostat (Leica CM-3050, Wetzlar, Germany).

Free-floating sections from each experimental group were processed for immunohistochemistry using an antibody against NPY (Affiniti Research Product, Exeter, UK). Sections were incubated with 1% normal goat serum for 2 hours to prevent nonspecific immunostaining. The sections were reacted with the primary antibody against NPY for 48 hours in 4°C, rinsed in PBS, and then incubated in biotinylated anti-rabbit immunoglobulin G for 2 hours at room temperature (RT). After rinsing them with PBS, sections were incubated in streptavidin-biotinylated peroxidase complex (Nichirei, Tokyo, Japan) for 2 hours at RT. The sections were rinsed in PBS and reacted

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Code numbers of primers and probes (Applied Biosystems)</th>
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<tr>
<td>5-hydroxytryptamine(serotonin)receptor 1A (5HT1AR)</td>
<td>Rn00561409_s1</td>
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<tr>
<td>5-hydroxytryptamine(serotonin)receptor 2A (5HT2AR)</td>
<td>Rn00568473_m1</td>
</tr>
<tr>
<td>NeuropeptideY (NPY)</td>
<td>Rn01410145_m1</td>
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<tr>
<td>Gamma-aminobutyric acid A receptor, alpha 1 (GABAΑ1R)</td>
<td>Rn00788315_m1</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
<td>Rn99999916_s1</td>
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with a solution containing 0.2 mg/mL 3,3'-diaminobenzidine tetra-hydrochloride and 0.06% hydrogen peroxide in 50 mM Tris-HCl buffer (pH 7.4) at RT, resulting in a brown precipitate.

**Statistical Analysis**

Values are expressed as means ± standard error of the mean. A paired *t*-test was used to compare the latencies or threshold values in behavioral tests between the ipsilateral side and the contralateral side (ECS group, *N* = 6; CCI group; *N* = 6). An unpaired *t*-test was used to compare the difference scores between the CCI group (*N* = 6) and the ECS group (*N* = 6). Dunnett’s test for multiple comparisons was used to compare latencies, threshold values, or difference scores obtained in behavioral tests performed before the operations (day 0, *N* = 6) with those obtained in tests performed after the operations (days 2, 4, 6, 8, 10, 12, and 14; *N* = 6).

Dunnett’s test was used to compare the gene expression levels for 5HT1AR, 5HT2AR, and GABA A R1 in whole brains of animals on days 14 after surgery (day 14) from the CCI group (*N* = 6) and the ECS group (*N* = 6) with those from the control group (*N* = 6). The SNK test was used to compare the gene expression levels for 5HT1AR, 5HT2AR, NPY, and GABA A R1 in whole brains of animals on day 14 among the CCI group (*N* = 6), the ECS group (*N* = 6), and the control group (*N* = 6). Dunnett’s test was used to compare the gene expression levels for NPY in whole brains of animals from the control group (*N* = 6) with those of the CCI group on days 1, 4, and 7 after surgery (day 1, *N* = 6; day 4, *N* = 6; day 7, *N* = 6). *P* < 0.05 was considered to be statistically significant.

**Results**

**Behavioral Tests**

Compared with the values on day 0, latencies of paw withdrawal from thermal stimulation (ECS group, *N* = 6; CCI group, *N* = 6; Figure 1A,B) and paw withdrawal thresholds in response to mechanical stimulation on the ipsilateral side (ECS group, *N* = 6; CCI group, *N* = 6; Figure 2A,B) were decreased on days 2, 4, 6, 8, and 10 (*P* < 0.01; Dunnett’s test). After ECS, the latencies of paw withdrawal from thermal stimulation on the ipsilateral side were increased on days 12 and 14 in the ECS group (*N* = 6; Figure 1A) and the difference score was significantly decreased (*P* < 0.01 compared with the CCI group; *N* = 6; unpaired *t*-test; Figure 1C). Paw withdrawal thresholds in response to mechanical stimulation were not significantly changed (ECS group, *N* = 6; CCI group, *N* = 6; Figure 2A,C) after ECS. ECS had no significant effect on the contralateral side.

**RT-PCR**

Expression ratios of NPY in the ECS group on day 14 were significantly greater than those in the CCI group (*P* < 0.05; SNK test; *N* = 6; Figure 3), and expression ratios of NPY in the CCI group were lower than those in the control group (*P* < 0.01; Dunnett’s test; *N* = 6; Figure 3). On the other hand, the expression of NPY in the ECS group was similar to that in the control group (Figure 3). Decreased NPY expression was observed in the CCI group of rats on days 1, 4, and 7 after surgery compared with the control group (*P* < 0.01; Dunnett’s test; *N* = 6; Figure 4). The expression levels for 5HT1AR, 5HT2AR, and GABA A R1 were not significantly different among the CCI group (*N* = 6), the ECS group (*N* = 6), and the control group (*N* = 6) (Figure 3).

**Immunohistochemistry**

There was no difference in NPY-immunostaining among the control group, the CCI group and the ECS group among the control group, the CCI group and the ECS group in the arcuate nucleus of the hypothalamus, in which wide distribution of NPY was observed (Figure 5). On the other hand, in the spinal trigeminal nucleus of the medulla, an increase of NPY-immunostaining was observed in the CCI group, and NPY-immunostainabilities in the control and ECS groups were similar (Figure 5).

**Discussion**

The results of this study show that ECS is effective for treating thermal hypersensitivity, but not for treating mechanical allodynia. This result is consistent with those of previous reports [14]. Up to postoperative day 10, the development of thermal hypersensitivity and mechanical allodynia was observed on the affected side in both the CCI and ECS groups, and a significant difference was confirmed between preoperative and postoperative data. Because there was no significant difference in the difference scores between the two groups, it can be said that there was no difference in the levels of CCI between the two groups. ECS had no effect on the contralateral hindpaw. Regarding the reason for the difference in efficacy of ECS for
thermal hypersensitivity and mechanical allodynia, Shibata et al. estimated that the central nervous system would have a large impact on thermal hypersensitivity, and that the nerves located lower than the spinal cord would affect mechanical allodynia largely; thus, ECS would have no effect on these lower nerves [14].

In the present study, RT-PCR results revealed a decrease in the level of expression of the NPY gene in rat brains in a model of neuropathic pain; however, after ECS, the expression of the NPY gene was increased to the level seen in the control group. Immunohistochemical staining showed that the expression of NPY was increased in the medulla in neuropathic pain model rats, and after ECS decreased to almost the same level as seen in the control group. The expression levels of the 5HT1A-R, 5HT2A-R, and GABA A,R genes were measured by RT-PCR, but no statistically significant differences were confirmed.

NPY is a neuropeptide that is widespread throughout the central nervous system, and is particularly located in the hypothalamus, cerebral cortex, and hippocampus. Although it is known that NPY is involved in the control of hypothalamic hormones and blood pressure, appetite and memory, it was also recently suggested that NPY could be related to pain [17–19]. NPY Y1 receptors are found in the midline raphe magnus [20], a key pain modulation center in the rostral ventromedial medulla (RVM), which contributes to the descending inhibitory system [21,22]. Taylor et al. showed that neuropathic pain was improved by administration of NPY to the RVM, and thus, it was supposed that NPY would have an inhibitory action on pain [17]. The results of the present study showed that NPY expression in the medulla is changed by chronic pain, but that it is restored to the same level as seen in the control group after ECS. This suggested a correlation between NPY levels and the effects of ECS on chronic pain.

Figure 1 Effects of electroconvulsive shock (ECS) administered to chronic constrictive injury (CCI) model rats (A) (N = 6) and 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. Difference scores were calculated as the value of the withdrawal latency on the ipsilateral side subtracted from the latency on the contralateral side (C) (the ECS group, N = 6; the CCI group, N = 6). The degree of reduction of thermal hyperalgesia by ECS is statistically significant (day 12, day 14). *P < 0.05, **P < 0.01 compared with values on the contralateral side on the same days by a paired t-test; ¶P < 0.05, ¶¶P < 0.01 compared with the CCI group by an unpaired t-test; #P < 0.05, ##P < 0.01 compared with the values on the preoperative day (day 0) by Dunnett’s multiple comparisons. Values are means ± standard errors of the mean.
Our immunohistochemical results contrasted with our RT-PCR results. Similar findings were shown in a previous report on the expression of vasopressin in the hypothalamus of animals with a salinity burden [23]. Based on the discussion in that report, we considered the following possible mechanisms to explain this discrepancy.

1. The intracellular expression of NPY mRNA may be increased by ECS.
2. The release of NPY might also be facilitated at the synapse owing to activation of axonal transport.
3. The rate of intracellular elimination of NPY caused by axonal transport was higher than the rate of NPY production caused by increased mRNA expression, and thus, the NPY level was decreased in immunohistochemical staining.

Figure 2 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. Difference scores were calculated as the value of the withdrawal threshold on the ipsilateral side subtracted from the threshold on the contralateral side (C) (the ECS group, N = 6; the CCI group, N = 6). ECS was not effective for treating mechanical allodynia. *P < 0.05, **P < 0.01 compared with values on the contralateral side on the same days, by a paired t-test; ***P < 0.05, ****P < 0.01 compared with the values on the preoperative day (day 0) by Dunnett’s multiple comparisons. Values are means ± standard errors of the mean.

Figure 3 Expression ratios of serotonin 1A receptor (5HT1AR), serotonin 2A receptor (5HT2AR), neuropeptide Y (NPY), and gamma-aminobutyric acid A α1 receptor (GABAα1R) in the brains of electroconvulsive shock (ECS) and chronic constrictive injury (CCI) group rats at 14 days after the CCI procedure. Expression ratios of NPY in the ECS group (N = 6) were significantly greater than those in the CCI group (N = 6). Expression ratios of NPY in the CCI group were significantly lower than those in the control group (N = 6). Expression ratios of NPY in the ECS group were similar to those in the control group. *P < 0.05 comparing the expression levels in the ECS group with those in the CCI group by SNK multiple comparisons test. **P < 0.05, ***P < 0.01 compared with the expression levels in the control group by Dunnett’s multiple comparisons. Values are means ± standard errors of the mean.
By contrast, in the neuropathic pain model, we estimated that the release of NPY at the synapse would be decreased and intracellular accumulation of NPY would be increased, thus inhibiting the expression of NPY mRNA.

Serotonin (5HT) is part of the descending inhibitory system in the central nervous system [24]. It has not yet been determined whether 5HT is inhibitory or facilitatory [24–27]. The results of our study showed no significant difference in the expression levels for the 5HT1A receptors and 5HT2A receptors after ECS.

GABA is also a component of the descending inhibitory system. There have been reports showing that the expression of GABA is increased in the occipital cortex of patients with depression when measurements were performed using MRS after ECT [28]. LaGraize and Fuchs showed that microinjection of GABA into the anterior cingulate gyrus attenuated place escape/avoidance behavior associated with mechanical stimulation, but did not decrease mechanical hyperalgesia or mechanical allodynia [29]. Therefore, it can be estimated that there may be a relationship between GABA levels and the efficacy of ECT. In the results of our study, however, no significant difference was confirmed in the expression of GABA receptor after ECS, although a tendency toward an increase in expression was observed. Because changes in genes were examined in the entire brain in this study, there was the possibility that such gene changes might be unclear and thus no significant difference was confirmed. Conversely, gene changes in brain regions that were not related to neuropathic pain might affect the study results. Thus, further research is required to examine which brain regions show the largest change in gene expression after ECS is administered for neuropathic pain.

In conclusion, we show that the symptoms of neuropathic pain are improved and the level of expression of the NPY gene in the brain is increased in CCI model rats after ECS. The possibility exists that changes in the levels of expression of NPY in the brain may be related to the

![Figure 4](image1.png) Expression ratios of neuropeptide Y (NPY) in the brains of chronic constrictive injury (CCI) model rats at 1, 4, and 7 days after the CCI procedure (N = 6 on all days). The expression ratios of NPY were significantly decreased on days 1, 4, and 7 compared with the control group (N = 6). *P < 0.01 compared with expression level in the control group by Dunnett’s multiple comparisons. Values are means ± standard errors of the mean.

![Figure 5](image2.png) Immunostaining for neuropeptide Y (NPY) in the arcuate nuclei of the hypothalamus in a rat undergoing no procedures (control group) (a), a chronic constrictive injury (CCI) model rat (CCI group) (b), a CCI model rat to which an electroconvulsive shock (ECS) was administered (ECS group) (c), and in the spinal trigeminal nucleus of the medulla of rats in the control group (d), CCI group (e), and ECS group (f). There was no difference in the levels of NPY among the CCI group, the ECS group, and the control group in the arcuate nuclei of the hypothalamus. In the spinal trigeminal nucleus of the medulla, the level of NPY was increased in the CCI group (arrows), while it was almost the same in the control and the ECS groups.
mechanism of action of ECT in treating neuropathic pain.

References


