Studies on the Changes of c-fos Protein in Spinal Cord and Neurotransmitter in Dorsal Root Ganglion of the Rat with an Experimental Peripheral Neuropathy

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Animal models for human chronic pain syndromes have been developed and widely used for pain research. One of these neuropathic pain models by Kim and Chung (1992) has many advantages for operation and pain elicitation. In this neuropathic model we have examined the c-fos protein, substance P, CGRP immunoreactivity in dorsal root ganglia and dorsal horn. 50 Sprague-Dawley rats were used for this study. L5 and L6 spinal nerves were ligated tightly to produce the neuropathic pain model. After 2, 4, 8, 16, and 24 hours and 1 week of surgery, rats were anesthetized and sacrificed by perfusion. After confirmation of the roccs transected by the surgery, the L5 and L6 dorsal root ganglia and spinal cord were removed and processed for immunohistochemistry. All tissue sections were immunohistochemically stained for substance P, CGRP and c-fos using the peroxidase-antiperoxidase (PAP) method. The number of immunostained substance P and CGRP dorsal root ganglion cells and c-fos immunoreactive dorsal horn cells were counted and analyzed statistically with Mann-Whitney U test.

The results are as follows. The number of c-fos protein immunoreactive neurons in the superficial layer of dorsal horn were increased markedly 2 hours after operation, and gradually decreased to normal level 1 week after operation. The number of c-fos protein immunoreactive neurons in the deep layer of the dorsal horn gradually increased to a peak 24 hours after operation, then decreased to the normal level 1 week after operation. The number of substance P and CGRP immunoreactive L5 and L6 dorsal root ganglion neurons were decreased markedly 1 week after the pain model operation.

In conclusion, after neuropathic pain model operation, c-fos proteins were immediately expressed in the superficial layer of spinal dorsal horn, thereafter c-fos proteins in the deep layer of spinal dorsal horn were expressed. CGRP and substance P immunoreactive neurons in DRG were decreased markedly 1 week after neuropathic pain model operation. These decrements do not coincide with the other chronic pain models, which show great increases in these pain transmitting substances. Therefore, the relationship between pain and c-fos, SP and CGRP should be investigated further.

Key Words: Pain, neuropathy, c-fos, substance p, CGRP, immunohistochemistry

INTRODUCTION

Pain is the most frequent complaint among patients, however, the mechanism of pain and its conducting pathway has not yet been elucidated. The precise mechanism of causalgia, one of the frequent syndromes after peripheral neuropathy, which was first described by Mitchell in 1872, is not yet known. Recently, an animal model for neuropathic pain was developed and research is actively being pursued.

There are several neurotransmitters, which are bases for the transmission of information in the nervous system. Since the immunocytochemical method using their antibodies was developed, many researchers have tried to understand the complex composition of the central nervous system. Generally it is accepted that the changes of neurotransmitter occur prior to the functional change of nervous system. It is probable that the pain conducting neurotransmitter is changed due to neuropathy after the injury of a peripheral nerve.
Substance P (SP), a peptide consisting of 11 amino acids, is the most probable candidate substance amongst pain conducting neurotransmitters.\textsuperscript{12,15,16} Substance gelatinosa, known as pain modulating region in the spinal cord is innervated with abundant SP containing nerve fibers.\textsuperscript{13,15,18} When capsaicin, the substance that interrupts pain transmission, has been administered, the concentration of substance P is decreased in the spinal cord,\textsuperscript{29,22} and there is much evidence indicating that SP is related to pain transmission physiologically.\textsuperscript{23-25} Based upon the above facts, there is a possibility that SP is a pain conducting neurotransmitter.

Calcitonin gene-related peptide (CGRP) is a neuropeptide which comes from the same loci as calcitonin\textsuperscript{26} and nerve fibers which contain CGRP are widely distributed in the various region. They are distributed in the Rexed lamina I and II in the spinal cord\textsuperscript{27-33} and are found only in unmyelinated C-fiber and thin myelinated A\delta fibers.\textsuperscript{32-34} Additionally, CGRP is the only neuropeptide which is found in the primary afferent fibers of the spinal dorsal horn\textsuperscript{30,35} and they are found in the same neurons in the dorsal root ganglia which contain SP.\textsuperscript{29} CGRP is also a candidate pain transmitting neuropeptide, and is found specifically in primary afferent fibers.\textsuperscript{31-33}

A c-fos, the proto-oncogene, which is cell p-fos, is found in murine osteogenic sarcoma virus, and is activated by growth factor and neurotransmitter in a few minutes.\textsuperscript{36} A c-fos is expressed in the spinal dorsal horn neurons after pain stimulation, and Hunt et al. (1987) reported the relationship between c-fos expression in the spinal dorsal horn and noxious stimulation, using c-fos mRNA product, and c-fos protein antigen.\textsuperscript{37-42} A c-fos gene product, c-fos protein is increased when pain is initiated, and the method of observing these phenomenon using immunohistochemistry has been widely used.\textsuperscript{43,44} Therefore, it is very interesting to observe changes in c-fos immunoreactivities.

Pain is conducted not only from the peripheral to the central nervous system, but is also modulated by descending influences. It is known that the descending tracts from periaqueductal gray, nucleus raphe magnus, gigantocellular reticular nucleus and locus ceruleus suppress pain.\textsuperscript{45,46}

The purpose of this study was to elucidate the effect of chronic neuropathy to neurotransmitters using immunohistochemistry and quantitative study using an image analyzer.

If the relation between neuropathy and neurotransmitters can be understood, the mechanism of neuropathy due to nerve injury will be better understood.

**MATERIALS AND METHODS**

**Experimental animals**

A total of 50 Sprague-Dawley rats weighing 200-300 gm were used in this study.

**Animal model**

All experimental procedures were performed on rats which were deeply anesthetized with sodium pentobarbital. For the neuropathy model, surgery was performed with Kim and Chung (1992)\textsuperscript{6} model. The left L5 and L6 spinal nerves were isolated and ligated tightly with 3-0 silk thread.

Animals were sacrificed on post operative days 1 and 7, and the spinal cords were stained immunohistochemically with CGRP and SP antibodies. In case of c-fos protein, immunohistochemical staining was done 2, 4, 8, 16 and 24 hours and 1 week after nerve ligation.

The tests for mechanical allodynia were performed in the 24 hr group and the 1 week group and only animals with mechanical allodynia were used for the experiment.

**Immunohistochemistry**

Animals were anesthetized with ether or sodium pentobarbital and perfused with saline followed by 3% paraformaldehyde-3% glutaraldehyde-0.1% picric acid solution. Spinal cord and spinal ganglia were dissected out under the stereomicroscope. Spinal cords were sectioned (50 \( \mu \)m) with a vibratome and immunostained for c-fos protein. DRG were sectioned serially and immunostained for SP and CGRP.

All tissue sections were stained immunocytochemically using the peroxidase-antiperoxidase
(PAP) method of Sternberger (1986). Sections were washed out with phosphate buffered saline (PBS) several times and incubated with 1% sodium borohydride for 1 hour. After washing with PBS, sections were incubated with 3% normal goat serum (NGS) for 30 minutes and incubated with CGRP, substance P and c-fos antibodies (Peninsular laboratories, Inc., Belmont, CA, U.S.A.) for 72 hours at 40°C. Triton X-100 was added to the antibody containing solutions to enhance antibody penetration. Following several rinses in phosphate buffer, the tissues were placed in 3% NGS for 30 min., followed by incubation with goat anti-rabbit gamma globulin (IgG) at a dilution of 1:50 for 1 h at room temperature. The sections were rinsed several times in phosphate buffer, and then placed in 3% NGS for 30 min., followed by incubation with PAP at a dilution of 1:100 for 1 h at room temperature. Sections were then rinsed several times in phosphate buffer and placed in 0.05% diaminobenzidine (DAB) solution containing 0.01% hydrogen peroxide for 10 min. Controls were prepared using incubating solution without primary antibodies.

Quantitative study

In dorsal root ganglia, neurons positively stained with CGRP and SP were counted in each section, and the overcounting error was corrected using the Konigsmark's formula (1970). In spinal cord dorsal horn, c-fos protein positive neurons were counted in the superficial layer (Rexed lamina I, II) and deep layer (Rexed lamina III, IV, V, VI), separately, and the results statistically analyzed using the Mann-Whitney U test.

RESULTS

Substance P (SP) neurons and CGRP neurons in dorsal root ganglia (DRG)

In both the control and experimental groups, SP and CGRP positive neurons were expressed with brown granules in the cytoplasm. SP positive neurons were distributed in the periphery of the ganglia and were mostly small B cells. CGRP positive neurons were distributed in the periphery of the ganglia, however, they were also found in the central region. CGRP positive cells were small B cells, but some of intermediate sized cells also showed CGRP immunoreactivities (Fig. 1, and 2). The SP and CGRP immunoreactive (IR) neurons of the experimental group showed no changes in the first 24 hours, but a severe decreased numbers and immunoreactivity were observed after 1 week (Fig. 3, and 4).

In L5 DRG, SP-IR neurons were not greatly changed statistically in the 24 hr group. However, in the 1 week group, the number of SP-IR neurons in the injured side was significantly diminished.

Fig. 1. Substance P (SP) immunoreactive neurons in the dorsal root ganglia of the 24 hr group (L5C: 5th lumbar DRG of the control side, L5L: 5th lumbar DRG of the lesion side).

Fig. 2. The SP immunoreactive neurons in the dorsal root ganglia of the 1 week group (L6C: 6th lumbar DRG of the control side, L6L: 6th lumbar DRG of the lesion side).

Fig. 3. The CGRP immunoreactive neurons in the dorsal root ganglia of the 24 hr group.

Fig. 4. The CGRP immunoreactive neurons in the dorsal root ganglia of the 1 week group.

L6 DRG showed a similar changing pattern (Table 1). In L5 DRG, CGRP-IR neurons were not dramatically changed in 24 hr group. However, in the
Table 1. The Number of Substance P Immunoreactive Cells of the Dorsal Root Ganglia after Nerve Injury

<table>
<thead>
<tr>
<th></th>
<th>24 hours after injury</th>
<th>1 week after injury</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control side</td>
<td>injured side</td>
</tr>
<tr>
<td>L5</td>
<td>1134 ± 379.7</td>
<td>888 ± 198.2</td>
</tr>
<tr>
<td>L6</td>
<td>704 ± 97.0</td>
<td>694 ± 88.9</td>
</tr>
</tbody>
</table>

1The numbers represent average standard deviation (S.D.).
2n=6 (24 hours after injury), n=5 (1 week after injury)
3n=6 (24 hours after injury), n=7 (1 week after injury)
*p<0.01 by Mann-Whitney U test to the control side.

Table 2. The Number of CGRP Immunoreactive Cells of the Dorsal Root Ganglia after Nerve Injury

<table>
<thead>
<tr>
<th></th>
<th>24 hours after injury</th>
<th>1 week after injury</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control side</td>
<td>injured side</td>
</tr>
<tr>
<td>L5</td>
<td>2900 ± 805.1</td>
<td>2176 ± 669.2</td>
</tr>
<tr>
<td>L6</td>
<td>1592 ± 395.5</td>
<td>1332 ± 296.0</td>
</tr>
</tbody>
</table>

1The numbers represent average standard deviation (S.D.).
2n=6 (24 hours after injury), n=5 (1 week after injury)
3n=6 (24 hours after injury), n=7 (1 week after injury)
*p<0.01 by Mann-Whitney U test to the control side.

Table 3. Changes of the Average Numbers of c-fos Protein Immunoreactive Neurons on the 5th Lumbar (L5) Superficial Dorsal Horn

<table>
<thead>
<tr>
<th></th>
<th>normal control</th>
<th>2 hours</th>
<th>4 hours</th>
<th>8 hours</th>
<th>16 hours</th>
<th>24 hours</th>
<th>1 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>control side</td>
<td>0.83 ± 0.18</td>
<td>4.26*</td>
<td>3.34*</td>
<td>3.27*</td>
<td>1.47*</td>
<td>1.83*</td>
<td>0.84</td>
</tr>
<tr>
<td>injured side</td>
<td>42.22*</td>
<td>25.99*</td>
<td>9.71*</td>
<td>4.30*</td>
<td>4.75*</td>
<td>4.75*</td>
<td>1.17</td>
</tr>
</tbody>
</table>

1The numbers represent average standard deviation (S.D.).
2n=5/group
*p<0.01 on Mann-Whitney U test to the control side.

The changes of c-fos protein immunoreactivity in the dorsal horn of the spinal cord

In the immunohistochemical preparation using c-fos protein antigen, the positive structures were expressed as dark brown granules in the nucleus of the spinal dorsal horn neurons (Fig. 5). In the control side, they were observed very rarely, being found mainly in the deep layer and were of a light brown color. In the experimental group, immunoreactive structures were darker and observed more often.

The superficial layer of the spinal dorsal horn

A c-fos protein activities were quantified by counting the immunoreactive nuclei in the spinal dorsal horn. Compared to the control side, c-fos protein immunoractive cells in the superficial layer, i.e. Rexed lamina I, II were increased from 2 hours after nerve injury and subsequently they decreased gradually. However, they maintained high levels for 24 hours later and this was statistically significant. One week after injury, they returned to normal levels (Table 3, and 4, Fig. 6, and 7).

Fig. 5. A c-fos protein immunoreactivities in the dorsal horn of spinal cord (L5). A c-fos protein immuno-positive structures presented as dark brown granules in the nucleus of the neurons (arrowed). Left side is the injured group and right side the control group.

Table 4. The Changes in the Average Numbers\(^3\) of c-fos Protein Immunoreactive Neurons on the 6th Lumbar (L6) Superficial Dorsal Horn

<table>
<thead>
<tr>
<th></th>
<th>normal control</th>
<th>2 hours</th>
<th>4 hours</th>
<th>8 hours</th>
<th>16 hours</th>
<th>24 hours</th>
<th>1 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>control side(^2)</td>
<td>0.81</td>
<td>3.27*</td>
<td>2.27*</td>
<td>2.82*</td>
<td>1.30</td>
<td>1.33</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>± 0.47</td>
<td>± 2.03</td>
<td>± 0.26</td>
<td>± 2.26</td>
<td>± 0.35</td>
<td>± 0.59</td>
<td>± 0.32</td>
</tr>
<tr>
<td>injured side(^2)</td>
<td>21.13*</td>
<td>14.62*</td>
<td>7.20*</td>
<td>3.20*</td>
<td>3.61*</td>
<td>1.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>± 6.25</td>
<td>± 4.45</td>
<td>± 1.93</td>
<td>± 0.40</td>
<td>± 1.08</td>
<td>± 0.07</td>
<td></td>
</tr>
</tbody>
</table>

\(^3\)The numbers represent average standard deviation (S.D.).
\(^2\)n=5/group
\(^*\)p<0.01 on Mann-Whitney U test to the control side.

Fig. 6. The changes in the average numbers of c-fos protein immunoreactive neurons on the 5th lumbar (L5) superficial dorsal horn (con: normal control).

On the other hand, in the control group, they increased for 24 hours, but to a lesser extent than the injured group (Table 3, Fig. 6).

In L6, the injured group showed an increasing pattern compared to the control group between 2 hr and 8 hr and this was statistically significant (Table 4, Fig. 7).

General pattern was similar in L5 and L6.
The deep layer of the spinal dorsal horn

In the deep layer of the spinal dorsal horn, Rexed lamina III, IV, V, VI, c-fos protein immunoreactivities increased gradually 24 hours after nerve ligation with a slight decrease in the 4 hr group, which normalized after 1 week (Table 5, and 6, Fig. 8, and 9). In the control side, c-fos protein immunoreactivities were increased in a similar manner to the injured side, however, the injured side showed a continuing increase, while the control side showed decrease from 16 hours.

Table 5. Changes in the Average Numbers\(^1\) of c-fos Protein Immunoreactive Neurons on the 5th Lumbar (L5) Deep Dorsal Horn

<table>
<thead>
<tr>
<th></th>
<th>normal control</th>
<th>2 hours</th>
<th>4 hours</th>
<th>8 hours</th>
<th>16 hours</th>
<th>24 hours</th>
<th>1 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>control side(^2)</td>
<td>4.12 ± 0.78</td>
<td>2.43</td>
<td>2.05</td>
<td>5.57*</td>
<td>4.92*</td>
<td>4.19</td>
<td>3.08</td>
</tr>
<tr>
<td>injured side(^2)</td>
<td>7.27* ± 1.96</td>
<td>4.29</td>
<td>7.74*</td>
<td>10.91*</td>
<td>11.47*</td>
<td>5.00</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)The numbers represent average standard deviation (S.D.).
\(^2\)n=5/group
\(^*\)p<0.01 on Mann-Whitney U test to the control side.

Table 6. Changes in the Average Numbers\(^3\) of c-fos Protein Immunoreactive Neurons on the 6th Lumbar (L6) Superficial Dorsal Horn

<table>
<thead>
<tr>
<th></th>
<th>normal control</th>
<th>2 hours</th>
<th>4 hours</th>
<th>8 hours</th>
<th>16 hours</th>
<th>24 hours</th>
<th>1 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>control side(^2)</td>
<td>3.11 ± 1.21</td>
<td>3.27</td>
<td>2.48</td>
<td>5.75*</td>
<td>4.50*</td>
<td>4.88*</td>
<td>2.90</td>
</tr>
<tr>
<td>injured side(^2)</td>
<td>3.98 ± 1.67</td>
<td>2.89</td>
<td>6.83*</td>
<td>7.85*</td>
<td>8.00*</td>
<td>3.79</td>
<td></td>
</tr>
</tbody>
</table>

\(^3\)The numbers represent average standard deviation (S.D.).
\(^2\)n=5/group
\(^*\)p<0.01 on Mann-Whitney U test to the control side.

Fig 8. Changes in the average numbers of c-fos protein immunoreactive neurons on the 5th lumbar (L5) deep dorsal horn (con: normal control).

Fig. 9. Changes in the average numbers of c-fos protein immunoreactive neurons on the 6th lumbar (L6) deep dorsal horn (con: normal control).
DISCUSSION

Causalgia, which was described by Mitchell in 1872, is the chronic pain syndrome that is produced after peripheral nerve injury, and is characterized by spontaneous pain, allodynia and hyperalgesia. It is known that this symptom is reduced by sympathectomy, however, the precise mechanism is not yet known.

Recently, animal models of peripheral neuropathy, caused by peripheral nerve injury have been developed, and used extensively. Among these, the Bennett & Xie model involves ligation of the sciatic nerve loosely and it is used widely, because of spontaneous pain and allodynia. However, the force of ligation cannot be the same in every experiment, so it is difficult to repeat the pain results. The model of Seltzer involves ligating the sciatic nerve 1/3 to 1/2 of the whole nerve strongly, which is also difficult to define, i.e., in terms of the forces employed of the part ligated. The Kim & Chung model involves ligation of the L5 and L6 dorsal roots forcefully and this technique is reproducible. In our experiments, after surgery, the animals showed symptoms of spontaneous pain, i.e., licking or biting feet and allodynia, characterized by avoiding behavior by innocuous stimulation with von Frey hair.

We used only animals that showed allodynia in the 24 hr and 1 week groups, as animals were not recovered from anesthesia at 16 hrs.

After pain stimulation, immediate early gene c-fos expression in the neurons of the spinal cord dorsal horn is observed. Hunt et al. (1987) verified that pain stimulation causes the expression of c-fos protein in the spinal dorsal horn immunohistochemistry, using c-fos antigen, the product of c-fos mRNA. Since that report, more papers have been published concerning noxious stimuli due to chemical substances, i.e. formalin or carrageenan or upon the continuous pain stimulation of arthritis, and found induced c-fos expression in spinal dorsal horn. On the other hand, innocuous stimulation does not express c-fos in the spinal dorsal horn. Presley et al. (1990) reported c-fos expression is reduced when morphine is administered systemically and Abbadie et al. (1992) reported that in neuropathic animals, various medications were administered and their effect was estimated by the animals level of reductance.

In this experiment, c-fos was expressed after surgery and c-fos protein positive neurons were increased significantly. This could be explained if pain stimulation is conducted via the spinal dorsal horn. However, other reports indicate that c-fos is expressed when the sciatic nerve is totally removed and facial nerve is resected out could not exclude that it is the effect of nerve resection. In our experiment, c-fos was expressed significantly, especially in the superficial layer, Rexed lamina I, II and these results coincide with other researcher’s results.

No reports about the changes in the deep layer of the spinal dorsal horn in the neuropathic pain model have been published to date. Hunt et al. (1987) reported that c-fos is expressed in the superficial layer only and no changes were observed in the deep layers and noted that there was some expression induced by non-noxious stimulation. Williams et al. reported that there was a significant increase in c-fos expression in the superficial layer 2 hr after nerve ligation and that this normalized in 8 hr, while in the deep layer the peak expression of c-fos was observed at 8 hr, and this persisted until the 24 hr even in the opposite side of the spinal cord.

Our results are similar to those of Williams et al’s, i.e. c-fos in the deep layer was expressed later than in the superficial layer and persisted longer, and that there was also significant c-fos increase in the opposite side. This verifies the explanation of Williams et al., namely, that there are molecular biological changes in the superficial layer initially and that these spread out to the deep layer after noxious stimulation. Our results also showed a slight decrease in the 4 hr group, which could not be explained.

In our experiment, the neuropathic model was a chronic pain model and allodynia was confirmed in the 24 hr and 1 wk groups, but c-fos expression in the spinal cord of these animals was at normal levels. A c-fos immunoreactivity in the control side was very lightly stained, while the injured side was significantly darker and remained quite different a week after insult. In addition, Bullitt et al. reported that they changed the noxious stimulation time from 3 to 24 hr and

that c-fos expression increased with stimulation time.\textsuperscript{65} It is believed that c-fos was not expressed because the noxious stimulation time was too short, for spontaneous pain persisted only a short duration in neuropathic pain. Therefore, to study the effects of drugs or electrical stimulation, the stimulation eliciting allodynia should be longer to properly allow comparisons of c-fos expression.

Immediate early genes like c-fos induced gene expression of neurotransmitter like SP and CGRP,\textsuperscript{12,13,16} known as substances for conducting pain in the dorsal horn of the spinal cord.\textsuperscript{12,13,16} Therefore, it is believed that this gene expression induces SP and CGRP synthesizing gene expression, and that increases in the levels of these substances makes the pain severe. However, our results show that SP and CGRP were not increased, but rather decreased after c-fos protein increase. Therefore, it is difficult to believe that these species are dependently related.

The SP and CGRP are normally increased in the spinal dorsal horn and in DRG during chronic pain, i.e. inflammations, such as those caused by arthritis or metabolic neuropathy. However, in the case of Ed-the highlight above-delete? neuropathic pain, SP and CGRP are decreased and there are many papers that report this reduction.

The reason for these reductions was the resection of L5 and L6 nerve roots, and these phenomena are not directly related to the mechanism of chronic pain.

Therefore, the relationship between pain and c-fos, SP and CGRP should be investigated further.

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