The effects of the sympathetic nerves on lumbar radicular pain
A BEHAVIOURAL AND IMMUNOHISTOCHEMICAL STUDY

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A rat model of lumbar root constriction with an additional sympathectomy in some animals was used to assess whether the sympathetic nerves influenced radicular pain. Behavioural tests were undertaken before and after the operation.

On the 28th post-operative day, both dorsal root ganglia and the spinal roots of L4 and L5 were removed, frozen and sectioned on a cryostat (8 µm to 10 µm). Immunostaining was then performed with antibodies to tyrosine hydroxylase (TH) according to the Avidin Biotin Complex method. In order to quantify the presence of sympathetic nerve fibres, we counted TH-immunoreactive fibres in the dorsal root ganglia using a light microscope equipped with a micrometer graticule (10 x 10 squares, 500 mm x 500 mm). We counted the squares of the graticule which contained TH-immunoreactive fibres for each of five randomly-selected sections of the dorsal root ganglia.

The root constriction group showed mechanical allodynia and thermal hyperalgesia. In this group, TH-immunoreactive fibres were abundant in the ipsilateral dorsal root ganglia at L5 and L4 compared with the opposite side. In the sympathectomy group, mechanical hypersensitivity was attenuated significantly.

We consider that the sympathetic nervous system plays an important role in the generation of radicular pain.

Radicular pain is one of the most common complaints treated by spinal surgeons. Mechanical compression of the nerve root and the chemical reactions induced by a herniated disc containing nucleus pulposus have been suggested as being the cause of sciatica, but the pathological mechanism is still unclear.1-12 Neuropathic pain after injury to a peripheral nerve is thought to consist of two types of pain, sympathetically maintained pain and sympathetically independent pain. In man, the former can be attenuated by sympathectomy or sympathetically maintained pain and sympathetically independent pain. In man, the former can be attenuated by sympathectomy or sympathetically maintained pain.13-15 In animal models of neuropathic pain caused by injury to a peripheral nerve, immunohistological studies have been performed to confirm that the sympathetic nervous system is involved in generating this. McLachlan et al16 found that sympathetic nerve fibres were increased in the corresponding dorsal root ganglion (DRG) after ligation of the sciatic nerve, sprouting to DRG somata and forming basket-like structures around axotomised sensory neurones of large diameter. This abnormal sympathetic-somatosensory interaction appears to lead to sympathetically maintained pain.16-18

The sympathetic nervous system is currently considered to be involved in causing radicular pain in man. Clinically, sympathetic block has been claimed to be effective for relieving this.19,20 In basic studies using animal models the influence of the sympathetic nervous system on radicular pain has not been fully explored.

Our aim was to elucidate the effects of the sympathetic nervous system on radicular pain. A lumbar radiculopathy model was used to investigate the pain-related behaviour. The distribution of sympathetic nerves around DRG neurones was examined using immunohistochemical analysis. In addition, after surgical sympathectomy had been performed on the lumbar radiculopathy model, the changes in pain-related behaviour were studied.

Materials and Methods
The experimental protocol used in this study was approved by the Sapporo Medical University Animal Care and Use Committee. We used a total of 59 male Sprague-Dawley rats weighing 150 g to 200 g at the beginning of the study.
Operative technique. All the surgical procedures were performed on rats anaesthetised intraperitoneally with sodium pentobarbital (50 mg/kg) under sterile conditions. They were placed in the prone position, and the L5-6 intervertebral space was identified. Through a midline dorsal incision from L4 to S1, the paraspinal muscles were retracted to expose the left L5-6 facet joint. A left L5 hemilaminectomy and L5-6 partial facetectomy were performed using a microscope. The size of the incision and exposure were minimised to avoid interference with DRG neurones as much as possible. In the root constriction group there were 14 rats, nine for the behavioural study and five for the immunohistochemical study. The left L5 spinal root was carefully exposed and then tightly ligated extradurally with an 8-0 nylon suture just proximal to the DRG. The surgical field was irrigated with sterile saline and closed with 4-0 monofilament sutures in layers. A total of 11 rats, six for the behavioural study and five for immunohistochemistry, had the same operative procedure, but without ligation of the nerve root (the sham group). A further ten rats (five each for behavioural and immunohistochemical studies) acted as a control group.

In another group of 14 rats, sympathectomy was performed immediately after root constriction. The animals were placed in the supine position and then, using a transperitoneal approach, the lumbar sympathetic chains were visualised by gently retracting psoas major. In identifying the levels of the sympathetic ganglia, the most reliable landmark was found to be the left renal artery which runs near the L2 ganglion. The L5 ganglion was usually located just proximal to the bifurcation of the descending aorta.21 Once each level had been identified, the sympathetic chains with the ganglia of both sides were resected from L2 to L5. A further ten rats only received the skin incision and exposure of the sympathetic chains, but no further surgery.

Behavioural study. Investigators blinded to the surgical protocol of each rat performed the behavioural tests. To assess the mechanical withdrawal response, the rats were placed in a Plexiglas chamber on a glass platform. The responses were measured as the latency of withdrawals of the hind paw elicited by a radiant heat source (Tail Flick Analgesia Meter; IITC Inc.,) which was moved beneath an area of the hind paw that was flush against the glass. The intensity of the heat stimulus was constant throughout all the experiments, and elicited a rapid withdrawal reflex with a latency of approximately six to eight seconds in the control rats. A cut-off time of ten seconds was set to prevent tissue damage. Each hind paw was tested five times at intervals of at least five minutes, alternating between the left and the right. The mean withdrawal latency was calculated from the last four measurements. Consequently, the thermal withdrawal latency of each rat was defined as the latency of the contralateral response (non-constricted) minus the ipsilateral response (constricted). Positive scores indicated increased and negative scores decreased sensitivity of the ipsilateral hind paw. The behavioural tests were performed two days before the operation and on the third, seventh, tenth, 14th, 21st and 28th post-operative day.

For both the sympathectomy and non-sympathectomy groups, mechanical stimulation of 4.5 g was applied to the plantar surface of the hind paw in the same way as in the root constriction model mentioned above. The withdrawal frequency was counted in each group. The behavioural tests were carried out before the operation and on the third, seventh, tenth and 14th post-operative days.

Immunohistochemistry. At the 28th post-operative day, the rats were killed. The DRGs and spinal roots of L4 and L5 were removed from each side, frozen and sectioned on a cryostat (8 µm to 10 µm). They were then immunostained with rabbit antibodies to tyrosine hydroxylase (TH; Chemicon, Temecula California) according to the Avidin Biotin Complex (ABC) method.22,23 This is an important enzyme which is synthesised by sympathetic nerve fibres. It transforms tyrosine, the precursor of noradrenaline, into dopa. Therefore, antibodies to TH are often used for assessing the presence of sympathetic nerve fibres. The sections were incubated with primary antibodies at a dilution of 1:1000 for 24 hours at room temperature (22°C), followed by incubation with biotinylated secondary antibody. The immunoreaction products were visualised by the diaminobenzidine (DAB) method24 in the presence of H2O2 in 0.1 M phosphate buffer. In order to quantify the extent of the presence of sympathetic nerve fibres, we counted TH-immunoreactive fibres in the DRG using a light microscope equipped with a micrometer graticule (10 x 10 squares,
We counted the squares of the graticule which contained TH-immunoreactive fibres for each of five randomly selected sections of DRG.

**Statistical analysis.** All the data were expressed as the mean ± SEM and all obtained from the observations of mechanical sensitivity and thermal withdrawal latency were analysed by two-way repeated measures of analysis of variance (ANOVA) at each time point, and then compared with the withdrawal difference from the pre-operative time point. The TH-immunoreactive fibres were analysed by one-way factorial measures of ANOVA in each group. Changes in the TH-immunoreactive fibres between the nerve root ligation side and the unligated side were compared by the unpaired, two-tailed *t*-test. A p-value < 0.05 was considered to be statistically significant.

**Results**

**Behavioural testing.** The withdrawal response frequencies of both paws were stable two days before the procedures. No significant differences in the baseline frequencies were observed in the different groups of rats. When stimulated with a 4.5 g filament, the root constriction group showed a gradual increase in tactile sensitivity from the third post-operative day, and a significant increase from the tenth day compared with the withdrawal difference from the pre-operative response (two way repeated measures ANOVA, *p* = 0.048) from the third to the 28th post-operative day compared with the pre-operative response and that in the control group (Fig. 1a).

The thermal withdrawal response was significantly faster (two way repeated measures ANOVA, *p* = 0.055) in the root constriction group compared with the pre-operative response and the response of the control group from the third to the 28th post-operative day. Root constriction resulted in mechanical allodynia and thermal hyperalgesia (Fig. 1b).

The results of the behavioural tests for mechanical sensitivity in the sympathectomy group are shown in Figure 2. The mechanical hypersensitivity observed in the non-sympathectomy group was attenuated significantly (two way repeated measures ANOVA, *p* = 0.0285) in the sympathectomy group. The effect of sympathectomy on radicular pain was seen from the third to the 14th day after operation.

**Immunohistochemical findings.** In the root constriction group, TH-immunoreactive fibres were more abundant in the ipsilateral L5 and L4 DRGs compared with the contralateral side. However, they could not be found around the DRG neurone soma, but were present around the myelin sheaths in the DRG (Fig. 3).

In the sham and control groups, TH-immunoreactive fibres were scarce in the L4 and L5 DRGs on both sides. There was no difference in their number between the ipsilateral and contralateral DRGs.

The mean number of the micrometer graticule squares containing TH-immunoreactive fibres in sections of the L5 DRG at the contralateral side (right), was $5.7 \pm 2.6$ in the 500 mm x 500 mm. We counted the squares of the graticule which contained TH-immunoreactive fibres for each of five randomly selected sections of DRG.
root constriction group, 4.8 ± 1.7 in the sham group and 4.5 ± 2.6 in the control group. There were no significant differences in any groups. On the ipsilateral side (left) of the L5 DRG, the mean number of squares was 23.6 ± 6.1 in the root constriction group, 7.2 ± 2.2 in the sham group and 4.8 ± 1.9 in the control group. TH-immunoreactive fibres increased significantly (one way factorial measures ANOVA, p < 0.0001) in the root-constricted ipsilateral (left) DRG (Fig. 4). At the ipsilateral L4 DRG, the number of positive squares was 12.1 ± 2.7 in the root constriction group, 4.7 ± 1.7 in the sham group and 5.3 ± 2.5 in the control group. Thus, the TH-immunoreactive fibres of the ipsilateral (left) L4 DRG increased significantly in the root-constricted DRG, although the reaction was less strong than in the L5 DRG.

Discussion

In the constriction model, mechanical allodynia and thermal hyperalgesia were observed on the planar aspect of the hind paw innervated with the involved L5 root, as described previously. When we applied mechanical stimulation to the hind paw, the withdrawal frequency increased gradually with the time from operation in the root constriction group. A significant increase was seen earlier when using the 11.3 g than with 4.5 g stimulus (Fig. 1a). The time for acquiring hypersensitivity depended on the intensity of stimulation. The withdrawal response to the noxious heat stimulus was also already present in the early post-operative period (Fig. 1b). These results are consistent with published data in other lumbar radiculopathy models.

We then examined the DRG neurones at the 28th post-operative day for sympathetic nerve fibres because the behavioural study had shown that mechanical allodynia and thermal hyperalgesia were observed up to this time after lumbar root constriction. The immunohistochemical investigation showed that TH-immunoreactive fibres were abundant in the corresponding DRGs at L5 compared with b) the contralateral (right) dorsal root ganglia.

Fig. 3a

Photomicrographs showing tyrosine hydroxylase immunohistochemistry. In the lumbar root constriction group, tyrosine hydroxylase-immunoreactive fibres (arrows) were more abundant around the myelin sheath in a) the ipsilateral (left) dorsal root ganglia at L5 compared with b) the contralateral (right) dorsal root ganglia.

Fig. 3b

Graph showing the time course of differences in withdrawal frequency in response to mechanical stimuli of 4.5 g for the ipsilateral versus the contralateral hind paws. The non-sympathectomy group showed hypersensitivity up to 14 days after surgery compared with the pre-operative response (*p < 0.05). Mechanical hypersensitivity was attenuated at each time point in the sympathectomy group. Each point represents the mean value of withdrawal frequency and each vertical bar the standard error of the mean.

Fig. 2
In general, it is considered that the sympathetic nervous system is important in the generation of pain. In low back pain, there is growing evidence that sympathetic afferents play a role. Nakamura et al. demonstrated by Cunha et al. that sympathetic adrenergic receptors in sympathetically maintained pain was demonstrated by Cunha et al. which was supported by a neuroanatomical study in the rat. They proposed a sympathetic afferent pathway which was supported by a neuroanatomical study in the rat and suggested that low back pain is a type of visceral pain. Based on neuroanatomical and immunohistochemical studies, it is also known that afferents from the lower lumbar disc have pathways to the DRG of L2 and are innervated by the lumbar sympathetic trunk. On the other hand, sympathetic efferents also have a role in low back pain. Neuroanatomical and immunohistochemical studies have revealed sympathetic neurones in the intervertebral disc and adjacent tissue. Based on neurophysiological studies, Roberts has proposed that the sympathetic system induces pain by activating Aβ afferents which excite wide dynamic range neurones in pain pathways in the spinal cord. Peripheral sensory nerves appear to have an increased concentration of alpha-adrenergic receptors after nerve injury. Elevated levels of norepinephrine may lead to phenotypic changes in nociceptors and ultimately to central sensitisation. Evidence for the role of beta-adrenergic receptors in sympathetically maintained pain was demonstrated by Cunha et al. by attenuation of inflammation-induced hyperalgesia by local injection of propranolol and atenolol. Gillette, Kramis and Roberts conducted a study on the lateral dorsal horn in cats while stimulating lumbar spine tissue and electrically stimulating the sympathetic chain. They found two patterns of response to sympathetic stimulation, entrained responses which appeared to be due to direct stimulation of afferents in the sympathetic trunk and non-entrained responses which were more variable in relation to a stimulus pulse and appeared to be caused by interaction between the noradrenergic sympathetic efferents and the primary afferents in the spinal tissue. The latter responses were blocked by the alpha-adrenergic antagonist, phentolamine. These results are consistent with the hypothesis proposed by Roberts that sympathetic efferents stimulate myelinated sensory fibres leading to stimulation of pain-producing wide dynamic range neurones in the dorsal horn of the spinal cord.

The effectiveness of sympathetic nerve block has been demonstrated clinically, not only for chronic pain such as causalgia, but also for lumbar radicular pain. Takahashi et al. found that sympathetic nerve block was still effective one month later in 59% of patients with leg pain resulting from stenosis of the lumbar spine canal who did not have relief from pain after epidural and lumbar root block. Yabuki and Kikuchi also observed that sympathetic nerve block was effective in approximately 40% of patients for relief from pain from radicular-type stenosis of the lumbar spinal canal.

In basic research using rats with peripheral nerve injury, McLachlan et al. reported that sympathetic nerve fibres increased in the corresponding DRGs after ligation of the sciatic nerve and sprouted to DRG somata, forming basket-like structures around axotomised sensory neurones of large diameter. The sympathetic nervous system connected directly to the somata of DRG neurones where sympathetic efferent signals were transmitted to secondary afferent neurones through DRG neurones. In our radiculopathy model, in which the nerve root was injured proximal to the DRG, sympathetic nerve fibres sprouted not to somata, but to myelin sheaths in DRG neurones. This implied that the sympathetic excitation must have indirectly influenced the DRG. Considering these pain models, different sprouting patterns of connection to the DRG may lead to clinical symptoms of differing severity, i.e., neuropathic pain, a refractory disease or radicular pain, with reversible symptoms.

In our study, TH-immunoreactive fibres were found to be increased not only at the corresponding DRG at L5 but also at the ipsilateral DRG at L4. It is considered that sympathetic sprouting is caused by many factors such as nerve growth factor, leukaemia inhibitory factor and interleukin (IL)-6. In particular, nerve growth factor is regarded as the most important factor for facilitating sprouting of sympathetic nerve fibres to the DRG after peripheral nerve injury, because the sympathetic neurones which sprout to form baskets express the high affinity nerve growth factor receptor, TrkA. In addition, anti-nerve growth factor

![Bar chart showing the mean numbers of squares containing tyrosine hydroxylase-immunoreactive (TH-IR) fibres in the dorsal root ganglia on both sides at L4 and L5. Tyrosine hydroxylase-immunoreactive fibres were increased significantly in the dorsal root ganglia at L4 and L5 in the root constriction, sham and control groups. Bars indicate the standard error of the mean. RT, right; LT, left.](image-url)
treatment can reduce injury-induced basket formation.\textsuperscript{46,47} Meanwhile, several compelling lines of evidence suggest that the uninjured L4 spinal nerve is the main route through which impulses evoked in the periphery are transferred to the spinal dorsal horn in models with ligation of the L5 root.\textsuperscript{48,49} Therefore in our rat model nerve growth factor may be synthesised in the L5 spinal nerve distal to the site of ligation, transported peripherally through spinal roots, diffused into the L4 spinal nerve, and transported retrogradely to the DRG neurones at L4. We hypothesise that this is the manner in which sympathetic nerve fibres are increased in the ipsilateral DRG at L4.

The difference between our radiculopathy model and peripheral nerve injury models is only that the site of injury is proximal or distal to the DRG. However, there may be different pathomechanisms of mechanical allodynia and thermal hyperalgesia between the root injury model and the peripheral nerve injury models. In the latter, it is considered that neuropeptides such as brain derived neurotrophic factor, calcitonin gene related peptide and substance P are transported anterogradely through spinal nerve roots from DRG neurones to the dorsal horn of the spinal cord.\textsuperscript{9,50,51} These neuropeptides play an important role in central sensitisation resulting in allodynia and hyperalgesia. In our radiculopathy model, injury proximal to the DRG influenced the transportation of neuropeptide from the corresponding DRG at L5 to the spinal dorsal horn. Thus, the mechanisms responsible for the development of mechanical allodynia and thermal hyperalgesia may be different in the root injury and peripheral nerve injury models. We consider that different sprouting patterns of sympathetic nerve fibres may be the key to these differences.

We have investigated the influence of the sympathetic nervous system on radicular pain by using immunohistochemical analysis. The TH-immunoreactive fibres were more abundant in the ipsilateral DRGs compared with contralateral DRGs in the root constriction group. The TH-immunoreactive fibres could not be found around the DRG neurone soma, as in peripheral nerve injury models, but were present around the myelin sheath in the DRG neurone. The mechanical hypersensitivity observed in the lumbar root constriction model was improved by sympathectomy. We suggest that the sympathetic nervous system has an important role in the generation of radicular pain.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References


