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Original Article

Investigation of sympathetic nerve branches in the pain conduction pathway of lumbar intervertebral discs in rats

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Abstract: Objective: To investigate the roles of the L2 and L5 sympathetic nerve branches in the conduction pathway of discogenic low back pain. Methods: One-hundred and twenty Sprague-Dawley rats were divided into the following groups: A, A-L_2, A-L_5, A-L_2-L_5, B (posterior intervertebral disc control), B-L_2, B-L_5, and B-L_2-L_5. The numbers of fluorogold (FG) and Substance P (SP) double-labeled cells in the DRGs of the T13, L1, L2, L3, L4, L5, and L6 segments were recorded. Results: The numbers of double-labeled DRG cells in the L2 segment were significantly lower in the A-L_2 and A-L_2-L_5 groups than in the group A ($P < 0.01$). Compared to the B group, the B-L_2 group had significantly fewer double-labeled DRG cells in the L2 segment ($P < 0.01$); the B-L_5 group had significantly fewer double-labeled DRG cells in the T13, L1, and L2 segments ($P < 0.01$) and the B-L_2-L_5 group had significantly fewer double-labeled DRG cells in the T13, L1, and L2 segments ($P < 0.01$). The numbers of double-labeled DRG cells in the L2 segment in the B-L_2-L_5 group were significantly lower than those in the B-L_2 and B-L_5 groups ($P < 0.01$). Conclusions: In the rat, the L2 sympathetic nerve branch participates in pain information conduction from the L5-6 intervertebral disc to the L2 DRG, while pain information from the posterior L5-6 intervertebral disc is conducted to the upper and lower lumbar vertebrae DRGs through two different pathways. The L5 sympathetic nerve branch is involved in the conduction pathway of the upper lumbar vertebra DRG.

Keywords: Sympathetic nerve branch, discogenic low back pain, conduction pathway, rat model

Introduction

Lower back pain is a common problem in humans, as about 50% to 80% of individuals have low back pain at some stage in their lives. In addition, 15% of individuals may develop chronic low back pain [1]. The number of patients who present to hospitals with low back pain is only second to that of patients with upper respiratory infections [2]. There is no consensus regarding the pathogenesis of low back pain. However, the most common cause of low back pain is lumbar intervertebral disc degeneration, which is a complex biological process influenced by the patient's environment, age, genetics, dynamics, and other many factors [3-5]. In recent years, some scholars have proposed a new concept of discogenic low back pain, to describe low back pain due to pathological changes in the internal composition and structure of lumbar intervertebral discs [6]. About 40% of patients with chronic lower back pain have discogenic low back pain [7].

At present, the understanding of the pathogenesis of discogenic low back pain is lacking. In patients with discogenic low back pain, the annulus fibrosus of the intervertebral disc has a pathological gap. Pain nerve fibers and torn vascular granulation tissue grow and along this gap as a part of a repair process. The structure of the intervertebral disc undergoes pathological changes during the above growth and repair process due to the actions of various inflammatory mediators, which in turn leads to low back pain [8, 9]. A magnetic resonance imaging study by Peng et al. [10] revealed that the high-intensity zone of the posterior lumbar disc in patients with discogenic low back pain was essentially the inflammatory granulation tissue of the annulus fibrosus cleft. Pain infor-

mation leads to pain sensation along the nerve conduction pathway of the central nervous system when a body part feels pain. Sympathetic nerves play important roles in the above conduction pathway [11]. The annulus fibrosus of the posterior intervertebral disc is dominated by a sympathetic nerve and a spinal nerve, and pain information is conducted by the sympathetic nerve [12]. Therefore, understanding the conduction pathway of discogenic low back pain is of great significance in clinical medicine.

Most previous studies on discogenic low back pain have mainly focused on the sympathetic trunk in animal models. However, severance of the sympathetic trunk has considerable effects on the abdominal organs and lower limb function in animals [13]. In this study, we used fluorogold (FG) retrograde tracing, and immunohistochemistry (IHC) for Substance P (SP) to preserve neurological function in the rats to the greatest extent possible. Pain information conduction pathways were studied after severance of the L2 and L5 sympathetic nerve branches of the frontal and posterior L5-6 intervertebral discs to investigate pathogenesis of discogenic lower back pain.

Materials and methods

Laboratory animals

One-hundred and twenty pure inbred Sprague-Dawley (SD) rats aged 6-13 weeks with body masses of 260-320 g were used in our study. The SD rats were purchased from Changsha Tianqin Biotechnology Co., Ltd, Changsha China. (SCXK (X) 2014-0010) and maintained in a clean environment with good ventilation, an indoor humidity of 51-56%, and a temperature of 20-25°C. The animals had free access to food and water. This animal experiment was approved by the hospital Ethics Committee.

Grouping and preparation

The rats were divided into the A, A-L_2, A-L_5, A-L_2-L_5, B, B-L_2, B-L_5, and B-L_2-L_5 groups based on similarity in body weight. Each group contained 15 rats. We intraperitoneally injected the rats with 3% pentobarbital at a dose of 40 mg/kg and fixed the arms and legs in a supine position after anesthesia. We then cut the abdomen under sterile conditions with an incision diameter of about 5 cm, longitudinally cut the peritoneum to dissociate it laterally, and fully exposed the location where the sympathetic trunk met the communicating branch. The sympathetic nerve branches of the intervertebral disc were not severed in groups A and B, the L2 sympathetic nerve branch of the frontal intervertebral disc was severed in the A-L_2 group, the L5 sympathetic nerve branch of the frontal intervertebral disc was severed in the A-L_5 group, and both the L2 and L5 sympathetic nerve branches of the frontal intervertebral disc were severed in the A-L_2-L_5 group. Similarly, the L2 sympathetic nerve branch of the posterior intervertebral disc was severed in the B-L_2 group, the L5 sympathetic nerve branch of the posterior intervertebral disc was severed in the B-L_5 group, and the L2 and L5 sympathetic nerve branches of the posterior intervertebral disc were severed in the B-L_2-L_5 group.

FG injection and treatment

FG (purchased from Shenzhen Otwo Biotechnology Co., Ltd, Shenzhen, China) was injected into the frontal and posterior L5-6 intervertebral discs of the rats. For FG injection into the frontal L5-6 intervertebral disc, the frontal L5-6 intervertebral disc of the rat was fully exposed above the abdominal aortic cross between the psoas muscle and the left side. The the frontal L5-6 intervertebral disc was then punctured to a depth of 1.5 mm using a syringe containing 0.2 μl of 10% FG, slowly injected the solution over 10 min, and closed the pinhole with cyanoacrylate adhesive to avoid the leakage of FG. For FG injection into the posterior L5-6 intervertebral disc, the communicating branch was treated carefully, the incision was sutured, and the rat was placed in a prone position. A longitudinal cut of 5 cm in length centered at the L5-6 interspinal interstice was made on the rat's back. The paravertebral muscle was peeled off, the superior borders of the L5 and L6 vertebral plates were removed, and the endorachis was exposed, and the endorachis was moved out to the contralateral midline. The annulus fibrosus was then punctured to a depth of 1 mm using a syringe containing 0.2 μl of 10% FG, slowly injected the solution over 10 min, and closed the pinhole with cyanoacrylate adhesive to avoid the leakage of FG. The incision was then closed with surgical suture silk after the FG injections into the frontal and pos-

Table 1. Rat General Conditions [n (%)] (x ± s)

Groups	n	Gender		Weekly age		Body mass (g)
		Male (%)	Female (%)	≤9 weeks	> 9 weeks	
A group	15	9 (60.00)	6 (40.00)	8 (53.33)	7 (46.67)	288.4±10.6
A-L_2 group	13	7 (53.85)	6 (46.15)	8 (61.54)	5 (38.46)	290.4±11.2
A-L_5 group	14	10 (71.43)	4 (28.57)	8 (57.14)	6 (42.86)	285.1±8.4
A-L_2-L_5 group	11	5 (45.45)	6 (54.55)	9 (81.82)	2 (18.18)	291.6±10.8
F/χ^2	-	0.865		2.458		0.989
P	-	0.595		0.449		0.405
B group	15	5 (33.33)	10 (66.67)	6 (40.00)	9 (60.00)	281.5±9.4
B-L_2 group	13	8 (61.54)	5 (38.46)	6 (46.15)	7 (53.85)	284.2±10.5
B-L_5 group	13	7 (53.85)	6 (46.15)	9 (69.23)	4 (30.77)	291.5±11.3
B-L_2-L_5 group	12	7 (58.33)	5 (41.67)	9 (75.00)	3 (25.00)	293.7±9.8
F/χ^2	-	2.751		4.758		4.283
P	-	0.425		0.182		0.207

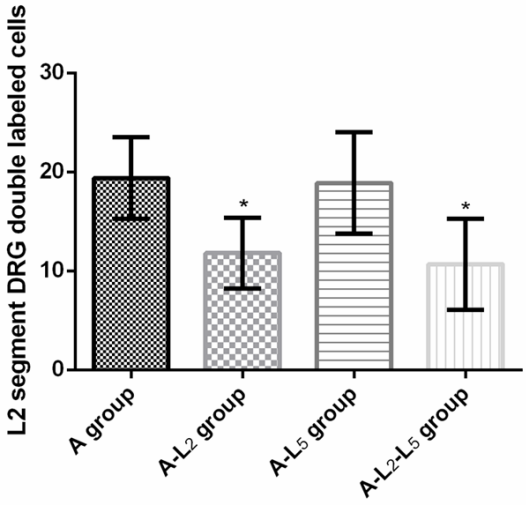

Figure 1. Double-labeled cell numbers in the L2 segment DRG. *P < 0.01, compared to group A.

terior intervertebral discs. The rats were injected with penicillin at a dose of 2,000,000 U/ml per day for 4 days after the operation to prevent infection. We euthanized the rats 7 days after the operation. At this time, the frontal and posterior T13 to L6 dorsal root ganglia (DRGs) were removed and the L5-6 intervertebral disc soft tissue. The DRGs and the intervertebral disc soft tissue were fixed and immersed them in a 20% sucrose solution in phosphate-buffered saline (PBS) overnight.

Immunohistochemical analysis

The DRGs were washed and embedded and produced 40-μm-thick long-axis DRG slices. The sections were incubated with 0.1% Triton X-100 solution for 20 minutes. The sections were incubated with rabbit anti-rat SP serum (provided by Abcam Trading Co., Ltd, Shanghai, China.) diluted 1:50 for 72 hours. The sections were then incubated in goat anti-rabbit secondary antibody diluted 1:100 for 60 minutes. The specimens were then washed with PBS and mounted on slides. The slides were then covered and sealed.

Screening and cell count

The L5-6 intervertebral disc were prepared and investigated the permeation of FG away from the injection site using an XSP-63X trinocular fluorescence microscope (Shanghai Optical Instrument Factory, Shanghai, China). FG oozing out of the annulus fibrosus was observed in the tissues surrounding the intervertebral disc and had spread to the central area of the nucleus pulposus. Fluorescent light microscopy revealed FG-positive cells with golden yellow cytoplasm, as well as SP-positive with bright red cytoplasm. We studied multiple slides at the same position and counted the double-labeled FG- and SP-positive cells.

Statistical method

SPSS 19.0 was used for statistical analysis. The data are reported as mean ± standard deviation (x ± s). Single-factor variance analysis was used for comparisons of means among the groups, Chi-square tests were used for comparisons of enumeration data among groups,

Table 2. Number of double-labeled cells before and after severance of L2 and/or L5 sympathetic nerves in the frontal L5-6 intervertebral disc in rats ($\bar{x} \pm s$)

Groups	N	T13	L1	L2	L3	L4	L5	L6
A group	15	4.1±2.8	14.8±4.8	19.4±4.1	6.4±2.5	5.4±3.1	2.8±1.2	4.3±1.8
A-L_2 group	13	4.2±2.0	14.5±3.9	11.8±3.6*	5.9±2.8	5.2±3.2	2.5±1.3	4.1±2.0
A-L_5 group	14	3.8±2.4	13.9±5.1	18.9±5.1	6.3±3.0	4.8±2.5	2.9±0.9	3.5±2.3
A-L_2-L_5 group	11	3.5±1.9	12.4±4.2	10.7±4.6*	5.8±3.1	4.5±2.1	3.0±1.4	3.7±1.9
F	-	0.221	0.659	14.250	0.140	0.265	0.404	0.460
P	-	0.880	0.581	< 0.001	0.935	0.849	0.750	0.711

Note: Compared to group A group, *P < 0.01.

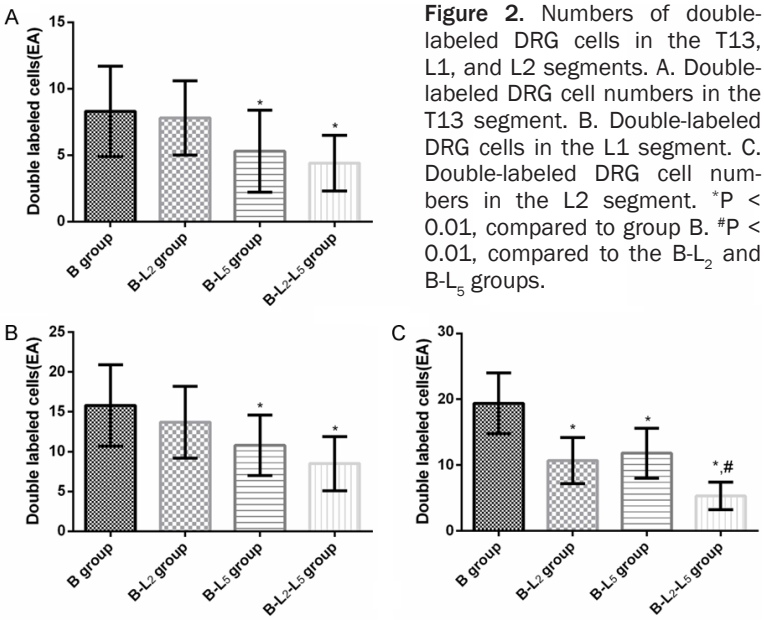

Figure 2. Numbers of double-labeled DRG cells in the T13, L1, and L2 segments. A. Double-labeled DRG cell numbers in the T13 segment. B. Double-labeled DRG cells in the L1 segment. C. Double-labeled DRG cell numbers in the L2 segment. *P < 0.01, compared to group B. #P < 0.01, compared to the B-L_2 and B-L_5 groups.

and least significant difference t tests were used for comparisons of intra-group data. Differences were considered statistically significant when *P* values were < 0.05.

Results

General condition of the rats

The coincidence rate in the A-L_2 group was 86.67% (13/15), as we failed to inject 2 rats with FG. The coincidence rate in the A-L_5 group was 93.33% (14/15), as we failed to inject 1 rat with FG; and the coincidence rate in the A-L_2-L_5 group was 73.33% (11/15), as we failed to inject 4 rats with FG. The coincidence rate was 86.67% (13/15) in both the B-L_2 and B-L_5 groups, as we failed to inject 2 rats with FG in each group. The coincidence rate in the B-L_2-L_5 group was 80.00% (12/15), as we failed to inject 3 rats with FG. There were no differences in the sex, age, or body mass of the rats in the different groups (P > 0.05) (**Table 1**).

Numbers of double-labeled cells before and after severance of the L2 and L5 sympathetic nerve branches of the frontal L5-6 intervertebral disc in rats

The numbers of double-labeled DRG cells in the L2 segment were significantly lower in the A-L_2 and A-L_2-L_5 groups than in group A (t = 5.173, P < 0.001; and t = 5.293, P < 0.001; respectively) (**Figure 1**). There were no significant differences in the numbers of double-labeled DRG cells in the other segments between the groups. There was no difference in the numbers of double-labeled DRG cells in the L2 segment or in other segments between the A-L_2 and A-L_2-L_5 groups (P > 0.05). There were no differences in the numbers of double-labeled DRG cells in the T13, L1, L2, L3, L4, L5, or L6 segments between the A-L_5 group and group A (P > 0.05) (**Table 2** and **Figure 1**).

Numbers of double-labeled cells before and after severance of the L2 and L5 sympathetic nerve branches of the posterior L5-6 intervertebral disc in rats

Compared to the group B, the B-L_2 group had significantly fewer double-labeled DRG cells in the L2 segment (t = 5.561, P < 0.001), the B-L_5 group had significantly fewer double-labeled DRG cells in the T13, L1, and L2 segments (t = 2.425, P = 0.022; t = 2.902, P = 0.007; and t = 4.720, P < 0.001; respectively), and the B-L_2-L_5

Table 3. Number of double-labeled cells before and after severance of L2 and/or L5 sympathetic nerve branches in the posterior L5-6 rat disc ($\bar{x} \pm s$)

Groups	N	T13	L1	L2	L3	L4	L5	L6
B group	15	8.3±3.4	15.8±5.1	19.4±4.6	12.6±3.8	11.2±2.7	8.9±3.4	4.2±2.7
B-L_2 group	13	7.8±2.8	13.7±4.5	10.7±3.5*	11.4±4.8	9.3±5.4	7.8±3.1	3.8±2.5
B-L_5 group	13	5.3±3.1*	10.8±3.8*	11.8±3.8*	11.7±5.1	10.3±3.8	9.0±4.6	4.1±3.2
B-L_2-L_5 group	12	4.4±2.1*	8.5±3.4*	5.3±2.1*,#	8.9±4.2	9.9±3.4	7.7±3.4	3.6±1.4
F	-	6.259	7.400	33.900	1.601	0.580	0.470	0.151
P	-	0.001	< 0.001	< 0.001	0.201	0.630	0.704	0.928

Note: Compared to group B, *P < 0.01; compared to the B-L_2 and B-L_5 groups, #P < 0.01.

group had significantly fewer double-labeled DRG cells in the T13, L1, and L2 segments (t = 3.472, P = 0.001; t = 4.613, P < 0.001; and t = 10.150, P < 0.001; respectively) (**Figure 2A-C**). There were no significant differences in the numbers of double-labeled DRG cells in other segments between the groups. The numbers of double-labeled DRG cells in the L2 segment were significantly lower in the B-L_2-L_5 group than in the B-L_2 and B-L_5 groups (t = 4.627, P < 0.001; t = 5.229, P < 0.001 respectively). There were no significant differences in the numbers of double-labeled DRG cells in the T13, L1, L2, L3, L4, L5, or L6 segments between the B-L_2 and B-L_5 groups (P > 0.05) (**Table 3** and **Figure 2A-C**).

Discussion

Lower back pain is primarily due to changes in the internal structures of intervertebral discs. Massive never fibers are contained in the outer layer of the lumbar intervertebral disc annulus fibrosus, and are distributed in the entire peripheral annulus fibrosus [9]. There are two types of pain transmission fibers: myelinated Aδ fibers, and unmyelinated C fibers [14]. Intervertebral discs can have an inflammatory response after disruption or injury of the annulus fibrosus. These responses may involve numerous inflammatory mediators and inflammatory cells. Varying degrees of lower back pain are triggered due to stimulation of internal pain receptors in the intervertebral disc nucleus pulposus and in the nerve endings of tissues surrounding the annulus fibrosus [15, 16]. SP-immunoreactive nerve fibers of normal intervertebral discs can exist in the outer layer of the annulus fibrosus, but SP-positive nociceptive nerve fibers are also observed in the degenerated nucleus pulposus and the interior intervertebral disc [17]. Mechanical or chemical substances that stimulate nociceptors or pain receptors can trigger lower back pain [18].

Pain information from lumbar intervertebral discs with pathological changes is conducted from the lumbar sinus vertebral nerve to the corresponding DRG. The corresponding neuron segment dominates the lumbar intervertebral disc [19]. However, discogenic low back pain is not distributed along the dominated regions of the surrounding tissues. Instead, it presents as disseminated back pain with less fixed tender points that are difficult to locate [20]. The haunch skin and lower waist are dominated by superior cluneal nerves, which are the posterior branches of cutaneous nerves in the L1-L3 segments. In contrast, no cutaneous nerves are distributed in the posterior branch regions of the S1 and L5 segments. Therefore, low back pain caused by pathological changes in lumbar intervertebral discs cannot be explained by radiative pain in the S1 and L5 segments, and is instead referred pain in the L1 and L2 segments [21]. The genitofemoral nerve is one of the branches of the DRG in the L2 segment and mainly carries pain information from the skin in the ventral groove region. Therefore, it is difficult to explain the domination of the nerve root ganglion segment [22].

A combination of FG retrograde tracking and immunohistochemistry for SP was used in this study to investigate the pain information pathways of the frontal and posterior L5-6 intervertebral discs in rats. FG had high sensitivity and can be used to label the cytoplasm. It is thus the most widely used neutral retrograde tracer. FG labels not only cells and nerve fibers transmitting pain, but also non-pain cells and fiber nerves [23]. SP is a neuropeptide involved in the transmission of pain information. SP-positive cells with bright red cyto-

plasms in the lumbar intervertebral disc represent DRG neurons [24]. There were no significant differences in the numbers of double-labeled DRG cells in any of the segments between the A-L_5 group and group A after severance of the L5 sympathetic nerve branch in the frontal L5-6 intervertebral disc in our rats. This indicates that the L5 sympathetic nerve branch did not participate in the nerve conduction pathway of the frontal L5-6 intervertebral disc in the rats. After severance of the L2 sympathetic nerve branch in the frontal L5-6 intervertebral disc in the rats, the A-L_2 and A-L_2-L_5 groups had significantly fewer double-labeled DRG cells in the L2 segment than group A. There were no significant differences in the numbers of double-labeled DRG cells in the L2 segment between the A-L_2 and A-L_2-L_5 groups. In addition, the function after the severance of only the L2 sympathetic nerve branch was similar to that after simultaneous severance of the L2 and L5 sympathetic nerve branches. The pain information conduction process in the frontal L5-6 intervertebral disc does not involve the L5 sympathetic nerve branch or DRGs in the same segment in rats. After severance of the L5 sympathetic nerve branch in the posterior L5-6 intervertebral disc, the numbers of double-labeled DRG cells were significantly reduced in the T13, L1, and L2 segments in the B-L_5 group when compared to group B. In addition, there were no significant differences in the numbers of double-labeled DRG cells in the other segments between the two groups. This indicates that pain information in the posterior intervertebral disc is conducted to the upper and lower lumbar vertebrae DRGs through two different pathways. The L5 sympathetic nerve branch is involved in the conduction pathway of the upper lumbar vertebra DRG, and is unrelated to the conduction pathway of the lower lumbar vertebra DRG. After severance of the L2 sympathetic nerve branch in the posterior L5-6 intervertebral disc, the numbers of double-labeled DRG cells were significantly reduced only in the L2 segment in the B-L_2 group when compared to group B. After simultaneous severance of the L2 and L5 sympathetic nerve branches, the numbers of double-labeled DRG cells in the L2 segment were significantly lower in the B-L_2-L_5 group than in the B-L_2 and B-L_5 groups. This indicates that both the L2 and L5 sympathetic nerve branches participate in and affect the conduction of pain information from the posterior L5-6 intervertebral discs to the L2 DRG.

This indicates that the L2 and L5 sympathetic nerve branches play different roles in the nerve conduction pathway of the L5-6 intervertebral disc in rats. A study by Takahashi et al. [25] reported the presence of sympathetic nerve fibers and receptor neuron fibers distributed in the lumbar intervertebral disc. The sensory nerve in the lumbar intervertebral disc enters the sympathetic nerve branch along the sinus vertebral nerve, but does not enter the spinal nerve in the same segment. Therefore, the L2 and L5 sympathetic nerve branches play different roles in the pain information conduction pathway in the L5-6 intervertebral disc in rats.

We ensured the reproducibility of the animal experiments in this study, and the rats purchased were carefully screened. In addition, there were no differences in sex, age, or body mass between the rats in the different groups, ensuring the reliability of the study. However, this study still has some shortcomings. First, it is difficult to assess the depth of the syringe when puncturing the intervertebral disc of the rats. This may easily lead to differences in the numbers of double-labeled DRG cells in the same segment. Second, the pain nerve conduction pathways are different between rats and humans. As such, this study does not shed light on processes underlying discogenic low back pain in humans. Third, we used normal rats for our study. As a result, we were unable to investigate specific mechanisms that may underlie the development of discogenic low back pain. The pain perception conduction pathway should thus be investigated in patients with pathological discogenic low back pain in a future study to further validate the findings of the present study.

In conclusion, the L2 and L5 sympathetic nerve branches play different roles in the conduction of pain information in the L5-6 intervertebral discs in rats. The L2 sympathetic nerve branch participates in the pain information pathway from the L5-6 intervertebral disc to the L2 DRG, while pain information in the posterior L5-6 intervertebral disc is conducted to the upper and lower lumbar vertebrae DRGs through two different pathways. The L5 sympathetic nerve branch is involved in the conduction pathway of posterior L5-6 intervertebral disc to the upper

lumbar vertebra and is unrelated to the conduction pathway of posterior L5-6 intervertebral disc to lower lumbar vertebra.

Disclosure of conflict of interest

None.

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