Eur Spine J (2003) 12:211–215
DOI 10.1007/s00586-002-0506-7

ORIGINAL ARTICLE

Seiji Ohtori
Kazuhisa Takahashi
Hideshige Moriya

Calcitonin gene-related peptide immunoreactive DRG neurons innervating the cervical facet joints show phenotypic switch in cervical facet injury in rats

Received: 21 January 2002
Revised: 6 July 2002
Accepted: 11 October 2002
Published online: 13 December 2002

S. Ohtori (✉) · K. Takahashi · H. Moriya
Department of Orthopaedic Surgery,
School of Medicine, Chiba University,
Chiba, Japan
e-mail: sohtori@ucsd.edu,
Tel.: +1-858-5343865,
Fax: +1-858-5341445

S. Ohtori
Anaesthesiology Research, 0629,
9500 Gilman Drive,
La Jolla, CA 92093–0629, USA

Abstract Patients with cervical facet lesions and facet joint injury sometimes experience diffuse neck pain, headache, and arm and shoulder pain. However, the pathophysiology of the intensity and expansion of facet joint pain has not yet been investigated. Retrograde transport of fluoro-gold (F-G) and immunohistochemistry of calcitonin gene-related peptide (CGRP) was used in 20 rats (control group, n=10; injured group, n=10). For the injured group, the whole facet capsule was incised. Of the total F-G labelled dorsal root ganglion (DRG) neurons innervating the C5/6 facet joint, the number and the cross-sectional area of cell profiles of F-G labelled CGRP-ir neurons were evaluated in the bilateral DRGs of both groups. The numbers of CGRP-ir F-G labelled DRG neurons as a percentage of all F-G labelled DRG neurons at C3, C4, C5, C6, C7, C8, T1, T2, and T3 respectively were 30, 22, 43, 47, 21, 19, 25, 36 and 30% in the control group, and 13, 15, 23, 17, 15, 8, 16, 28 and 35% in the injured group, with the injured group showing a significantly lower percentage of CGRP-ir F-G labelled neurons than the control group at C5 and C6 (P<0.05). However, the mean cross-sectional area of F-G labelled CGRP-ir cells from C3 to C8 DRGs increased from 625±22 μm^2 to 878±33 μm^2 in the injured group (P<0.001). Associated with the injured facet joints, the phenotypic switch to large neurons may complicate the mechanism of injured facet pain.

Keywords Calcitonin gene-related peptide · Ganglia · Spinal · Joint capsule · Neck pain

Introduction

Several studies have reported the cervical facet joints as a possible source of neck pain [2, 4, 5, 7, 8, 19]. Morphologically, the joint capsule is well innervated, receiving nerve supply from the medial branches of the dorsal rami [4]. Each medial branch segmentally innervates at two facet joints [4].

It has been reported that the C5/6 facet joint is multisegmentally innervated by C3, C4 and C7–T3 DRGs through paravertebral sympathetic trunks and by C5 and C6 DRGs directly through the medial branches of the dorsal rami [13].

It has also been reported that Substance P (SP) and calcitonin gene-related peptide (CGRP) immunoreactive (ir) nerve fibres are present within the facet joint capsules [1, 7, 21]. SP and CGRP are markers of sensory neurons involved mainly in pain perception [6]. We have also reported earlier on the existence of CGRP-ir DRG neurons innervating the C5/6 facet joint by retrograde neurotransport method and immunohistochemistry [14].

Previous clinical studies have reported that patients with cervical facet joint lesions and cervical facet joint injury sometimes experience diffuse neck pain, headache, and arm and shoulder pain [2, 5, 7, 8, 19]. However, the mechanism of the intensity and expansion of facet joint pain has not been investigated.

The present study was undertaken to determine the distribution and phenotypic changes of CGRP-ir DRG neurons innervating the injured C5/6 facet joint in rats. The C3, C4 and C7–T3 DRGs innervating the C5/6 facet joints through paravertebral sympathetic trunks and the C5 and C6 DRGs innervating the C5/6 facet joints directly through the medial branches of the dorsal rami have been separately discussed.

Materials and methods

Twenty male Sprague-Dawley (SD) rats weighing 250–300 g were used. They were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.) and treated aseptically throughout the experiments. A midline dorsal longitudinal incision was made over the cervical spine. The left C5/6 facet joint capsule was exposed under a microscope. A 26-G needle whose tip was filled with two fluoro-gold crystals (F-G; fluorochrome, Denver, Colo.) was advanced into the facet joint. The hole was immediately sealed with cyanoacrylate to prevent leakage of F-G. Then the fascia and skin were closed.

For the injured model, 2 weeks after application of F-G, 10 of the 20 rats were anaesthetized again, and the capsule of the same facet joint was cut completely.

Three weeks after the first surgery, these 20 rats were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.) and perfused transcardially with 0.9% saline, followed by 500 ml of 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Bilateral DRGs from the C3 to T3 levels were resected. The specimens were immersed in the same fixative solution overnight at 4°C. After storing in 0.01-M phosphate buffer saline (PBS) containing 20% sucrose for 20 h at 4°C, each DRG was sectioned at 40 μm thickness on a cryostat. We analysed all the sections that were obtained – around 20 from each DRG.

Immunohistochemistry of CGRP

Sections of DRGs were collected in PBS. Endogenous tissue peroxidase activity was quenched by soaking the sections for 30 min in 0.3% hydrogen peroxide solution in 0.01 M PBS. The specimens were then treated for 90 min in blocking solution, 0.01 M PBS containing 0.3% Tritron X-100 and 1% normal goat serum, at room temperature. They were processed for CGRP immunohistochemistry by free-floating ABC technique using rabbit antibody for CGRP (1:2000; Chemicon, Temecula, Calif.) diluted with a blocking solution for 20 h at 4°C, biotinylated goat anti-rabbit IgG (1:100; Vector Labs, Burlingame, Calif.), and fluorescein iso-thiocyanate (FITC) avidin D (1:100; Vector Labs, Burlingame, Calif.). After each step, the sections were rinsed in 0.01 M PBS three times. The sections were observed with a fluorescent microscope. The number of F-G labelled neurons and of F-G labelled CGRP-ir neurons in all the sections was counted. The cross-sectional areas of cell profiles of F-G labelled CGRP-ir neurons from C3 to C8 DRGs were also measured in the bilateral DRG neurons in both groups with a computer-assisted imaging analysis system (NIH Image software ver 1.58). The procedure was performed by a blinded observer.

Statistical analysis

The data were compared by ANOVA. A P-value of less than 0.05 was considered statistically significant.

Results

F-G labelled DRG neurons

DRG neurons labelled by F-G transported from the C5/6 facet joint were present in the left DRGs from C3 through T3 in the control and the injured groups (Table 1). No labelled neurons were observed in the contralateral DRGs from C3 through T3. The segmental distribution of the F-G labelled neurons is shown in Table 1. The F-G labelled neurons were small, intermediate, and large. The number of F-G labelled neurons in the DRGs in C5 was significantly greater than that in other DRGs in both the control and the injured group (Table 1) ($P<0.05$). Of the F-G labelled neurons, 41% in the control group and 42% in the injured group were recognized in the C5 and C6 DRGs, and the remaining 59% in the control group and 58% in the injured group were identified in the C3, C4, and C7–T3 DRGs. There were no differences in distributional pattern and ratios of F-G labelled neurons in DRG from C3 to T3 between control and injured group.

Table 1 Calcitonin gene-related peptide-immunoreactive (CGRP-ir) dorsal root ganglia (DRG) neurons labelled by fluoro-gold (F-G) innervating the left C5/6 facet joint. The numbers of F-G labelled CGRP-ir DRG neurons compared to all F-G labelled DRG neurons are presented for C3 through to T3 in the form of ratios and as percentages (e.g. 1/5 indicates a total of five F-G labelled DRG neurons, 1 of which was an F-G labelled CGRP-ir neuron

	Left side									Right side	Total
	C3	C4	C5	C6	C7	C8	T1	T2	T3		
Ratio (%) of F-G labelled CGRP-ir neurons in control group (n=10)	6/22 (30%)	9/41 (22%)	45/105 (43%)*	19/40 (47%)*	10/47 (21%)	6/32 (19%)	5/21 (25%)	9/24 (36%)	8/19 (40%)	0/0 (0%)	117/35 (34%)
Ratio (%) of F-G labelled CGRP-ir neurons in injured group (n=10)	3/24 (13%)	7/48 (15%)	25/95 (23%)*	10/55 (17%)*	7/44 (15%)*	5/36 (8%)	3/18 (16%)	7/24 (28%)	5/14 (35%)	0/0 (0%)	75/358 (19%)

* $P<0.05$

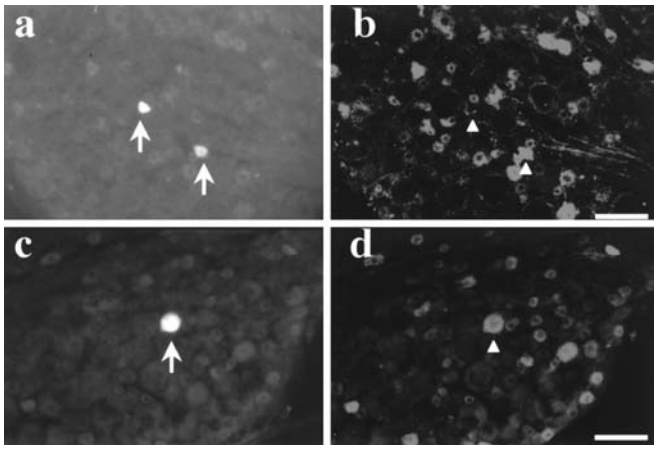


Fig. 1 Fluorescent photomicrographs of the control group (**a**,**b**) and the injured group (**c**,**d**) at the left C5 level. **a,c** Fluoro-gold (F-G) labelled dorsal root ganglia (DRG) neurons innervating the C5/6 facet joint. **b,d** Calcitonin gene-related peptide-immunoreactive (CGRP-ir) neurons labelled by fluorescein iso-thiocyanate (FITC) avidin D. The *arrows* in **a** and **c** show the same neurons as are shown by *arrowheads* in **b** and **d** (*bar*=100 μm)

Table 2 Mean (±SE) cross-sectional (CS) area (in μm^2) of cell profiles of F-G labelled CGRP-ir DRG neurons at each level

Level of DRG	Control group		Injured group	
	No. of cells	CS area	No. of cells	CS area
C3, C4, C7, C8	30	612±35*	22	982±39*
C5, C6	60	632±28**	34	761±47**
Total	90	625±22*	56	878±33*

*P<0.001; **P<0.05

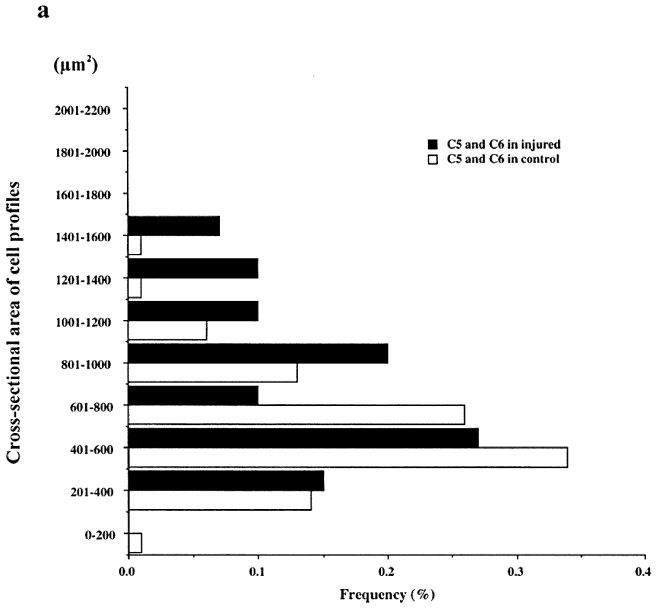
F-G labelled CGRP-ir neurons

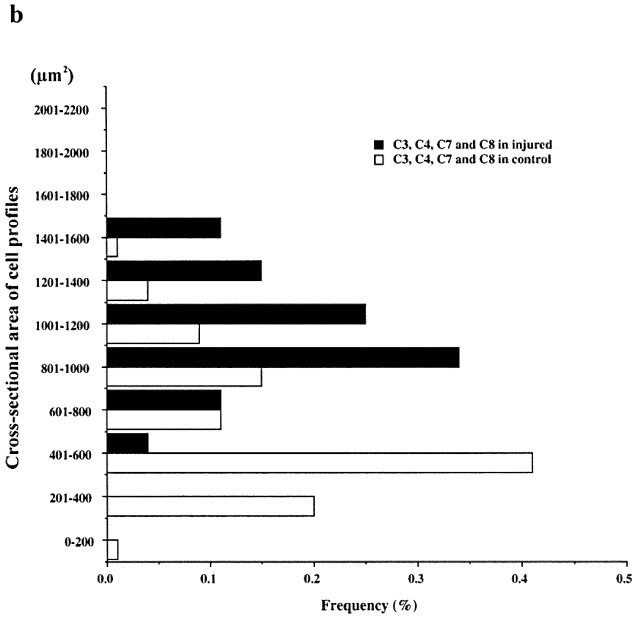
CGRP-ir neurons labelled by F-G were present in the left DRGs from C3 through T3 (Table 1). Figure 1 shows the F-G labelled CGRP-ir DRG neurons in both groups. The differences between the control group and the injured group with respect to the ratios of F-G labelled CGRP-ir neurons to all F-G labelled neurons were not significant for the C3, C4 and C7–T3 DRGs, but there were significant differences for the C5 and C6 DRGs (P<0.05).

Cross-sectional area of cell profiles from C3 to C8 DRGs

A total of 90 F-G labelled CGRP-ir neurons from C3 to C8 DRGs were measured in the control group, and a total of 56 were measured in the injured group. The cross-sectional areas of cell profiles of F-G labelled CGRP-ir neurons at each level in both groups are shown in Table 2 and Fig. 2. The mean cell profile from C3 to C8 DRGs was from 175 to 1450 μm^2 (625±22 μm^2; mean±SE) in the control group (C3, C4, C7, and C8, 612±35 μm^2; C5 and C6, 632±28 μm^2; mean±SE). The mean cell profile from C3 to C8 in the injured group was from 325 to 1550 μm^2 (878±33μm^2; mean±SE) (C3, C4, C7 and C8, 982±39 μm^2; C5 and C6, 761±47 μm^2; mean±SE). The mean F-G la-

Fig. 2a–b Size-frequency histogram illustrating the distribution of the cross-sectional areas of the profiles of F-G labelled CGRP-ir DRG neurons (90 neurons in the control group, 56 neurons in the injured group). Injury resulted in an overall shift in the distribution of the profile size to a larger size. Large neurons which do not produce CGRP under normal condition start to generate the product in the injured condition (**a** C5 and C6 DRG neurons; **b** C3, C4, C7 and C8 DRG neurons)





belled CGRP-ir cell profile of C3, C4, C7, and C8 DRGs and that of C5 and C6 DRGs was significantly greater in the injured group than in the controls ($P<0.001$).

Discussion

Innervation pattern of C5/6 facet joint (F-G labelled neurons)

It has been reported that in the rat, the C1/2, C3/4, and C5/6 facet joints are innervated by two distinct systems [11]. In one system, sensory nerve fibres from the facet joint are believed to pass via the medial branch of dorsal rami and reach DRGs at the adjacent level. In the other system, sensory nerve fibres enter the paravertebral sympathetic trunks through the ramus communicans and reach the distant DRGs directly via each ramus [13]. The C1/2, C3/4, and C5/6 facet joints are mostly innervated by the DRG at the adjacent level [13]. In the current study, the pattern of sensory innervation was the same; the C5/6 facet joint was multi-segmentally innervated by DRGs. Furthermore, the facet joint was more richly innervated by C5 DRG neurons than other DRGs. Although, there are seven facet joints in the cervical spine, we chose the C5/6 facet joint in this study. This was because it is one of the most clinically common symptomatic joints in humans [2, 8, 19]. As in humans, rats have seven cervical vertebrae, so the rat C5/6 facet joint appears to correspond to the human C5/6 facet joint [13]. In the current study we only examined the C5/6 facet joint. If the pattern of sensory innervation in the rat corresponds to that in humans, we can extrapolate the findings from the experimental model to further understand the clinical cervical facet syndrome, in which patients experience diffuse neck pain and headache [2, 5].

CGRP-ir DRG neurons labelled by F-G in the control group

CGRP is present in about 45% of the primary sensory neurons in DRG [6]. DRG neuronal size is correlated with the calibre of primary afferent axons, and usually CGRP is localized to small neurons, which are usually associated with unmyelinated axons [6]. These axons terminate mainly in laminae I and II of the spinal dorsal horn and carry information from peripheral nociceptors [6]. Medium and large cells in the DRG have myelinated axons that carry information from low-threshold mechanoreceptors, and terminate mainly in laminae III and IV of the spinal dorsal horn [22]. A study of electrophysiologically characterized neurons in lumbar dorsal root ganglia using dye injection revealed CGRP-like immunoreactivity in 46% of C-fibre neurons, 33% of A-delta fibre neurons, and 17% of A-alpha/beta fibre neurons [10]. These data show that CGRP is mainly a marker of pain perception, although under some circumstances CGRP is a marker of proprioception. Recently, using immunohistochemical techniques, several authors have reported CGRP-ir nerve fibres in the facet joint [1, 7, 18], and under physiological conditions in rats, CGRP-ir neurons innervating cervical facet joints were characterized as small DRG neurons [14]. In the current study, we did not examine the electrophysiological characteristics of neurons. However, anatomical measurement of cell size (from 175 to 1450 μm^2) leads us to conclude that most CGRP-ir DRG neurons transmit pain sensation, not proprioception or other modalities. Nevertheless, some FG-labelled CGRP-ir neurons are perhaps not involved in nociception.

Phenotypic switch of CGRP-ir DRG neurons labelled by F-G in the control group to larger neurons

Large DRG neurons send A-beta fibres to laminae III and IV of the spinal cord, an area that is considered to participate in the processing of innocuous input, such as that from brush and pressure [20]. After peripheral nerve injury, allodynia and hyperalgesia are not only the result of peripheral sensitization [16], but also the consequence of altered sensory processing in the spinal cord. Central sensitization in spinal cord is a facilitation of synaptic transmission following an increase in afferent fibre activity [3, 21]. Morphological study revealed that A-delta fibres sprout into laminae I and II from laminae III and IV after sciatic nerve axotomy [22, 23], and that the sprouted terminals establish synapse-like structures with neurons in the substantia gelatinosa (lamina II) [9].

It has been demonstrated electrophysiologically that, following sciatic nerve transection, large myelinated A-beta afferent fibres establish synaptic contact with interneurons and transmit innocuous information to substantia gelatinosa [15].

After peripheral nerve injury, substance P (SP) and CGRP in the small DRG neurons decreases [11, 12]. However, SP and CGRP, which are not produced in medium and large DRG neurons under physiological conditions, start to be expressed in these neurons [11, 14]. The expression of SP and CGRP in large DRG neurons is induced by mechanical allodynia [11, 12]. These results indicate that a subpopulation of DRG neurons express CGRP in response to peripheral nerve injury, and transport this peptide to laminae III and IV in the spinal dorsal horn. The increase in CGRP in the deep laminae may affect the excitability of postsynaptic neurons, and may have a role in neuronal plasticity following peripheral nerve injury [11].

In the peripheral nerve injured group, the ratios of F-G labelled CGRP-ir neurons decreased in the C5 and C6 DRGs. However, injury resulted in an overall shift in the distribution of the size of profiles to the larger size, and the mean cross-sectional area of the cell profiles was also enlarged in C3, C4, C7, and C8 DRG neurons. Large

DRG neurons sending their proximal axons to laminae III and IV appear to start producing CGRP, and to release this peptide to the laminae III and IV, sensitizing the interneurons and secondary neurons. The number of CGRP-ir neurons in the C5 and C6 DRGs decreased, but the number in C3, C4, C7, and C8 DRG neurons did not change. The reason for this remains unclear. However, it has been reported that some neurons innervating the rat lumbar facet joint were surrounded by CGRP-ir sensory fibres in the paraventral sympathetic trunks, and may be affected by these CGRP-ir fibres [17]. We were led to suspect that the respective pathways may be affected in different ways after nerve injury.

Limitations of the study

Limitations of the current study include:

1. We did not evaluate pain behaviour because, while it generally is possible to examine pain behaviour originating from rat foot, it is impossible to evaluate rat neck.
2. We did not examine the electrophysiological phenotype of the changed neurons.
3. Changes in the spinal dorsal horn were not examined.

Conclusions

In previous clinical reports, patients with cervical facet lesions and facet injury sometimes experience severe pain that may extend over a large area [2, 5]. Our results suggest that after injury, large neurons, which do not normally produce CGRP, start to generate it. These findings may provide a basis for the pathophysiology of pain perceived by humans after cervical facet lesions and facet joint injury.

Acknowledgement We thank Miss Keiko Kitajo for her expert technical assistance.

References

1. Ashton IK, Ashton BA, Gibson SJ, Polak JM, Jaffray DC, Eisenstein SM (1992) Morphological basis for back pain: the demonstration of nerve fibers and neuropeptides in the lumbar facet joint capsule but not in ligamentum flavum. J Orthop Res 10:72–78
2. Barnsley L, Lord SM, Wallis BJ, Bogduk N (1995) The prevalence of chronic cervical zygapophysial joint pain after whiplash. Spine 20:20–25
3. Basbaum AI, Chi SI, Levine JD (1992) Peripheral and central contribution to the persistent expression of the C-fos proto-oncogene in spinal cord after peripheral nerve injury. In: Willis WD (ed) Hyperalgesia and allodynia. Raven Press, New York, pp 295–304
4. Bogduk N (1982) The clinical anatomy of the cervical dorsal rami. Spine 7: 319–330
5. Bogduk N, Marsland A (1988) The cervical zygapophysial joints as a source of neck pain. Spine 13:610–617
6. Hökfelt T (1991) Neuropeptides in perspective: the last ten years. Neuron 7: 867–879
7. Inami S, Shiga T, Tsujino A, Yabuki T, Okado N, Ochiai N (2001) Immunohistochemical demonstration of nerve fibers in the synovial fold of the human cervical facet joint. J Orthop Res 19: 593–596
8. Kaneoka K, Ono K, Inami S, Hayashi K (1999) Motion analysis of cervical vertebrae during whiplash loading. Spine 24:763–769
9. Lekan HA, Carlton SM, Coggeshall RE (1996) Sprouting of A fibers into lamina II of the rat dorsal horn in peripheral neuropathy. Neurosci Lett 208:147–150
10. McCarthy PW, Lawson SN (1990) Cell type and conduction velocity of rat primary sensory neurons with calcitonin gene-related peptide-like immunoreactivity. Neuroscience 34:623–632
11. Miki K, Fukuoka T, Tokunaga A, Noguchi K (1998) Calcitonin gene-related peptide increase in the rat spinal dorsal horn and dorsal column nucleus following peripheral nerve injury: upregulation in a subpopulation of primary afferent sensory neurons. Neuroscience 82:1243–1252
12. Noguchi K, Kawai Y, Fukuoka T, Senba E, Miki K (1995) Substance P induced by peripheral nerve injury in primary afferent sensory neurons and its effect on dorsal column nucleus neurons. J Neurosci 15:7633–7643
13. Ohtori S, Takahashi K, Chiba T, Yamagata M, Sameda H, Moriya H (2001) Sensory innervation of the cervical facet joints in rats. Spine 26:147–150
14. Ohtori S, Takahashi K, Moriya H (2002) Calcitonin gene-related peptide immunoreactive sensory DRG neurons innervating the cervical facet joints in rats. J Orthop Sci 7:258–261
15. Okamoto M, Baba H, Goldstein PA, Higashi H, Shimoji K, Yoshimura M (2001) Functional reorganization of sensory pathways in the rat spinal dorsal horn following peripheral nerve injury. J Physiol 532:241–250
16. Peal ER (1992) Alterations in the responsiveness of cutaneous nociceptors: sensitization by noxious stimuli and the induction of adrenergic responsiveness by nerve injury. In: Willis WD (ed) Hyperalgesia and allodynia. Raven Press, New York, pp 59–79
17. Suseki K, Takahashi Y, Takahashi K, Chiba T, Tanaka K, Moriya H (1996) CGRP-immunoreactive nerve fibers projecting to lumbar facet joints through the paravertebral sympathetic trunk in rats. Neurosci Lett 221:41–44
18. Suseki K, Takahashi Y, Takahashi K, Chiba T, Tanaka K, Morinaga T, Nakamura S, Moriya H (1997) Innervation of the lumbar facet joints. Spine 22: 477–485
19. Speldewinde GC, Bashford GM, Davidson IR (2001) Cervical zygapophyseal joint blocks for chronic cervical pain. Med J Aust 174:174–176
20. Willis WD, Coggeshal RE (1991) Sensory mechanism of the spinal cord. Plenum Press, New York, pp 153–212
21. Woolf CJ (1983). Evidence for a central component of post-injury pain hypersensitivity. Nature 306:686–688
22. Woolf CJ, Shortland P, Coggeshall RE (1992) Peripheral nerve injury triggers central sprouting of myelinated afferents. Nature 355:75–78
23. Woolf CJ, Shortland P, Reynolds M, Ridings J, Doubell T, Coggeshall RE (1995) Reorganization of central terminals of myelinated primary afferents in the rat dorsal horn following peripheral axotomy. J Comp Neurol 360:121–134