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# Fifth lumbar spinal nerve injury causes neurochemical changes in corresponding as well as adjacent spinal segments: A possible mechanism underlying neuropathic pain

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ABSTRACT

Previous investigations of the anatomical basis of the neuropathic-like manifestations in the spinal nerve ligation animal model have shown that the central terminations of the unmyelinated primary afferents of L5 spinal nerve are not restricted to the corresponding L5 spinal segment, and rather extend to two spinal segments rostrally and one segment caudally where they intermingle with primary afferents of the adjacent L4 spinal nerve. The aim of the present study was to investigate the neurochemical changes in the dorsal horn of the spinal cord and DRGs after L5 nerve injury in rats. In the first experiment, the right L5 nerve was ligated and sectioned for 14 days, and isolectin B4 (IB4, a tracer for unmyelinated primary afferents) was injected into the left L5 nerve. The results showed that the vasoactive intestinal peptide (VIP) was up-regulated in laminae I–II of L3–L6 spinal segments on the right side in exactly the same areas where IB4 labelled terminals were revealed on the left side. In the second experiment, L5 was ligated and sectioned and the spinal cord and DRGs were stained immunocytochemically with antibodies raised against various peptides known to be involved in pain transmission and hyperalgesia. The results showed that L5 nerve lesion caused down-regulation of substance P, calcitonin-gene related peptide and IB4 binding and up-regulation of neuropeptide Y and neurokinin-1 receptor in the dorsal horn of L4 and L5 spinal segments. Similar neurochemical changes were observed only in the corresponding L5 DRG with minimal effects observed in L3, L4 and L6 DRGs. Although, L5 nerve injury caused an up-regulation in NPY, no change in SP and CGRP immunoreactivity was observed in ipsilateral garcile nucleus. These neuroplastic changes in the dorsal horn of the spinal cord, in the adjacent uninjured territories of the central terminations of the adjacent uninjured nerves, might explain the mechanism of hyperalgesia after peripheral nerve injury.


## 1. Introduction

The precise mechanisms that underlie neuropathic pain are not fully understood. Potential explanations are numerous (Costigan et al., 2009; Devor, 2006; Saadé and Jabbur, 2008; Sandkühler, 2009; Yaksh and Sorkin, 2005), but there seems to be a general consensus that pain originates from a lesion in the nervous system (Campbell and Meyer, 2006). However, although lesions of the central nervous system may lead to pain, most of the available models for neuropathic pain (Campbell and Meyer, 2006; Ossipov et al., 2006) are based on investigations carried out after peripheral nerve injury (Bennett and Xie, 1988; Decosterd and Woolf, 2000; Kim and Chung, 1992; Seltzer et al., 1990). Spinal nerve ligation (SNL) is a commonly used model to produce neuropathic-like manifestations in rodents. In this model, when the fifth lumbar (L5) spinal nerve is ligated and cut, rats develop hyperalgesia and allodynia in the hind paw (Kim and Chung, 1992).

Studies have shown that peripheral nerve injury results in substantial changes in the expression of neuropeptides, enzymes and *Bandeiraea simplicifolia* isolectin B4 (IB4) binding in the primary afferent neurons (Hökfelt et al., 1994, 1997; Navarro et al., 2007). In 1979, Jessell et al. were the first to show down-regulation of Substance P (SP) in dorsal horn after sciatic nerve injury. Later, data from our laboratory (Shehab and Atkinson, 1986a,b) and others (McGregor et al., 1984) showed that sciatic nerve section caused down-regulation of SP, somatostatin, cholecystokinin and fluoride resistant acid phosphatase (FRAP) and up-regulation of vasoactive intestinal polypeptide (VIP). Furthermore, we showed that the up-regulation of VIP in the dorsal horn after sciatic nerve section was of primary afferent origin (Shehab and Atkinson, 1986b) and more specifically from the injured neurons located in the dorsal root ganglia (Shehab et al., 1986). Subsequently, other investigators showed down-regulation in calcitonin gene related peptide (CGRP) (Dumoulin et al., 1991; Noguchi et al., 1990; Zhang et al., 1995b),

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*isolectin B4* (IB4) (Molander et al., 1996; Shehab et al., 2003, 2004; Tajti et al., 1988) and up-regulation in neuropeptide Y (NPY) (Shehab et al., 2003, 2004; Wakisaka et al., 1991; Zhang et al., 1995b), galanin (Hökfelt et al., 1987; Villar et al., 1989) and neurokinin-1 receptor (NK1r) (Goff et al., 1998) in the territories where sciatic nerve is known to terminate in the dorsal horn of the spinal cord. Although the significance of the neurochemical changes after nerve injury is not very well understood, some of those changes, e.g. the VIP up-regulation, might be involved in the regeneration or compensatory process following peripheral nerve injury (Hökfelt et al., 1994, 2006; Navarro et al., 2007), while others are likely to modify pain transmission. In keeping with this, it is reported that up-regulation in galanin inhibits the increased nociceptive input after peripheral nerve section (Wiesenfeld-Hallin et al., 1992). In case of NPY, which is up-regulated after nerve injury, the situation appears more complex. Intrathecal administration of NPY in normal rats produces antinociceptive action but in the flexor reflex model it shows a pronociceptive effect (Hökfelt et al., 2006). With regard to NK1r, the close relationship between the neuropathic pain behaviour and its up-regulation after nerve injury and inflammation suggest that substance P (through its receptor; NK1r) may act as an important mediator of hyperalgesia (Abbadie et al., 1996; Goff et al., 1998; Khasabov et al., 2002; King et al., 2005; Mantyh and Hunt, 2004; Nichols et al., 1999).

Previous works in which lesion of the dorsal root and Golgi techniques were used in cats (Szentagothai, 1964) or IB4 as a marker for unmyelinated nerve fibres in rats (Shehab et al., 2008) provided the anatomical basis of the neuropathic-like manifestations in SNL model. Such studies showed that the central terminations of the unmyelinated primary afferents are not restricted to the corresponding spinal segment that they enter. The central terminations of the unmyelinated primary afferents of L5 spinal nerve in rats extend to two spinal segments rostrally (L3 and L4) and one segment caudally (L6) (Shehab et al., 2008). Shehab et al. (2008) also showed that the central terminations of L4 spinal nerve have a similar pattern of distribution to the one found in L5, although they are displaced rostrally by one segment i.e. central terminals were observed in the dorsal horn of L4 and extended into two rostral spinal segments (L2 and L3) and one segment caudally (L5) with the densest labelling in L3 and L4. Importantly, Shehab et al. (2008) also found that the central terminations of unmyelinated primary afferents of the L4 and L5 spinal nerves intermingle with primary afferents of the adjacent spinal nerves in the dorsal horn at the L3–L5 levels. Therefore, it is predicted that neurochemical changes after L5 nerve injury would not only take place at the L5 spinal segment where the L5 spinal nerve enters the spinal cord but also in the rostral and the caudal segments which receive primary afferent inputs from the uninjured adjacent nerve. Consequently, the aim of the present study was to investigate the effects of L5 nerve ligation cut on the distributions of CGRP, SP and its receptor NK1r, IB4 binding, VIP and NPY in the dorsal horns of L3, L4, L5 and L6 spinal segments and the corresponding dorsal root ganglia (DRGs). CGRP, SP and IB4 were used as normal contents of unmyelinated and presumably thin myelinated primary afferents while VIP and NPY were used as markers for injured unmyelinated (and presumably thin myelinated) and myelinated primary afferents respectively. Part of this study had appeared in a previous abstract (Shehab, 2011).

## 2. Materials and methods

### 2.1. Surgical procedures

#### 2.1.1. Experiment I: spinal nerve ligation and IB4 injection

In this experiment a comparison between the locations of VIP up-regulation in the dorsal horn of the spinal cord (after right L5 injury for 14 days) and the central terminations of the contralateral L5 nerve (revealed three days after IB4 injection into the left L5) was investigated. The reason for leaving the animals for 14 days was due to previous observations that VIP up-regulation was maximal after this postoperative period of time following sciatic nerve injury (Shehab and Atkinson, 1986a,b). Adult Wistar rats (210–245 g) were anaesthetised with a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) delivered intraperitoneally. The skin of the back was incised longitudinally and the paravertebral muscles retracted. To reveal the L5 spinal nerve the transverse processes of the sixth lumbar vertebra was excised. In this experiment, the right L5 was ligated with a 6/0 silk suture and sectioned distally and the muscles and the skin were sutured in layers ($n = 4$). After 11 days of recovery the rats were reanesthetized again and the left L5 spinal nerve was exposed and injected with 1 μl of IB4 (2%, Vector) through a fine glass micropipette. Three days after the injection of IB4, the animals were deeply anesthetized with an overdose of urethane (2 ml, 25%) injected intraperitoneally and perfused through the ascending aorta with Zamboni's fixative (10% formalin containing 15% of saturated picric acid) in 0.1 M phosphate buffer (pH 7.4). The spinal lumbar segments from L3 to L6 were dissected out, postfixed in the same fixative for 3–4 h, and stored in 30% sucrose in phosphate buffer overnight. Left L3–L6 dorsal root ganglia (DRGs) were also dissected out and postfixed in the same fixative for another 1 h and then stored in 20% sucrose in phosphate buffer (pH 7.4) overnight.

#### 2.1.2. Experiment II: spinal nerve ligation

Rats were anaesthetized and the left L5 spinal nerve was ligated and cut. After 1 ($n = 3$), 7 ($n = 4$) and 14 ($n = 6$) days of recovery the animals were perfused with Zamboni's fixative and spinal cord segments, brainstem and DRGs were dissected out and postfixed as mentioned above.

### 2.2. Immunocytochemistry

#### 2.2.1. Spinal cord

Transverse sections (50 μm) of L3–L6 spinal segments were cut in a cryostat and were treated with 50% ethanol to enhance antibody penetration. The sections were then processed for immunofluorescence staining according to the protocols described previously (Shehab et al., 2003, 2004, 2008). Details of the primary antibodies used, their sources, and concentrations are given in Table 1. Spinal cord sections from animals in experiment I were incubated overnight with a mixture of rabbit anti-VIP and goat anti-IB4. After rinsing, the sections were incubated for 1 h with a mixture of donkey anti-rabbit IgG conjugated Rhodamine Red (1:100; from Jackson ImmunoResearch) and donkey anti-goat IgG conjugated to Alexa 488 (1:200; Molecular Probe/Invitrogen). Sections from animals in experiment II were incubated overnight with one or two of the following antibodies: VIP, SP, NK1r, CGRP

**Table 1**
Primary antibodies used in this study.

| Antibody | Species | Dilution | Source |
|---|---|---|---|
| Substance P | Rabbit | 1:5000 | Bachem/Peninsula Lab |
| Substance P | Rat | 1:200 | Immunological Direct |
| NK1r | Rabbit | 1:5000 | Professor Couraud |
| CGRP | Rabbit | 1:5000 | Bachem/Peninsula Lab |
| CGRP | Sheep | 1:5000 | Affiniti Research Products |
| NPY | Goat | 1:5000 | Affiniti Research Products |
| VIP | Rabbit | 1:5000 | Professor J. Allen |
| IB4 | Goat | 1:1000 | Vector Laboratories |
| ATF3 | Rabbit | 1:200–1:2000 | Santa Cruz |