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Research Report

Unmyelinated primary afferents from adjacent spinal nerves intermingle in the spinal dorsal horn: A possible mechanism contributing to neuropathic pain

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ABSTRACT

Peripheral nerve injury in animals can cause neuropathic pain often expressed in the form of hyperalgesia and allodynia. Spinal nerve ligation, in which the fifth and sixth lumbar (L5 and L6) or only the L5 spinal nerve is ligated and cut, is a model commonly used to produce neuropathic pain. The purpose of the present study was to test whether there is any anatomical evidence to support the suggestion that terminating unmyelinated (C) fibres of injured and adjacent uninjured nerves interact at the level of the spinal dorsal horn. Thus, in the first series of experiments, rats received injections of anterograde tracers, either wheat germ agglutinin (WGA) conjugated to horseradish peroxidase or *Bandeiraea simplicifolia* isolectin B4 (IB4), into the L4 or L5 spinal nerves. Results with both tracers showed that the central terminals of nerve L4 were concentrated in both L4 and L3 segments of the dorsal horn with clear although reduced levels of labelling in L2 and L5. Similarly, the central terminals of nerve L5 were found in both L5 and L4 again with less labelling in L3 and L6. These results suggest an intermingling of primary afferents of adjacent nerves at the level of the spinal dorsal horn. A second series of experiments was therefore conducted to test whether primary afferent terminals from adjacent nerves target the same neuronal elements in the regions of overlap. Consequently, additional rats were injected with WGA into the L5 spinal nerve and IB4 into the adjacent L4 spinal nerve. Double immunofluorescent staining and confocal microscopy revealed that IB4-labelled and WGA-labelled boutons, belonging to L4 and L5 spinal nerves, terminated in the same region within the L4 spinal segment. This suggests that neurons located in regions of overlap receive input from both L4 (intact) and L5 (injured) afferents. Consequently, spinal neurons located in regions of terminal overlap may show augmented responses to activation of the intact L4 nerve due to neuronal sensitisation resulting from injury to the adjacent L5 nerve. This may in part provide an anatomical basis for hyperalgesic reaction to injury.

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1. Introduction

The mechanisms that underlie neuropathic pain are complex and poorly understood. Various animal models of neuropathic pain have recently been developed (Bennett and Xie, 1988; Seltzer et al., 1990; Kim and Chung, 1992; Decosterd and Woolf, 2000). These models have been generally based on production of peripheral nerve injuries (Ossipov et al., 2006). Spinal nerve ligation (SNL) is one of the animal models commonly used to produce neuropathic pain. In this model, either the fifth and sixth lumbar (L5 and L6) (Kim and Chung, 1992) or only the L5 spinal nerve is ligated and cut, following which rats develop long-lasting ipsilateral hyperalgesia and allodynia in the foot, similar to that seen in human neuropathic pain. Whether injured or neighbouring uninjured nerves are responsible for the production of neuropathic pain in this model is debatable (Ringkamp and Meyer, 2005). The role of different classes of primary afferents in neuropathic pain is also controversial (Ringkamp and Meyer, 2005). However, in a recent review, Campbell and Meyer (2006) discussed the role of central sensitisation in the production of neuropathic pain. They predicted that "the injured L5 afferents could also project to

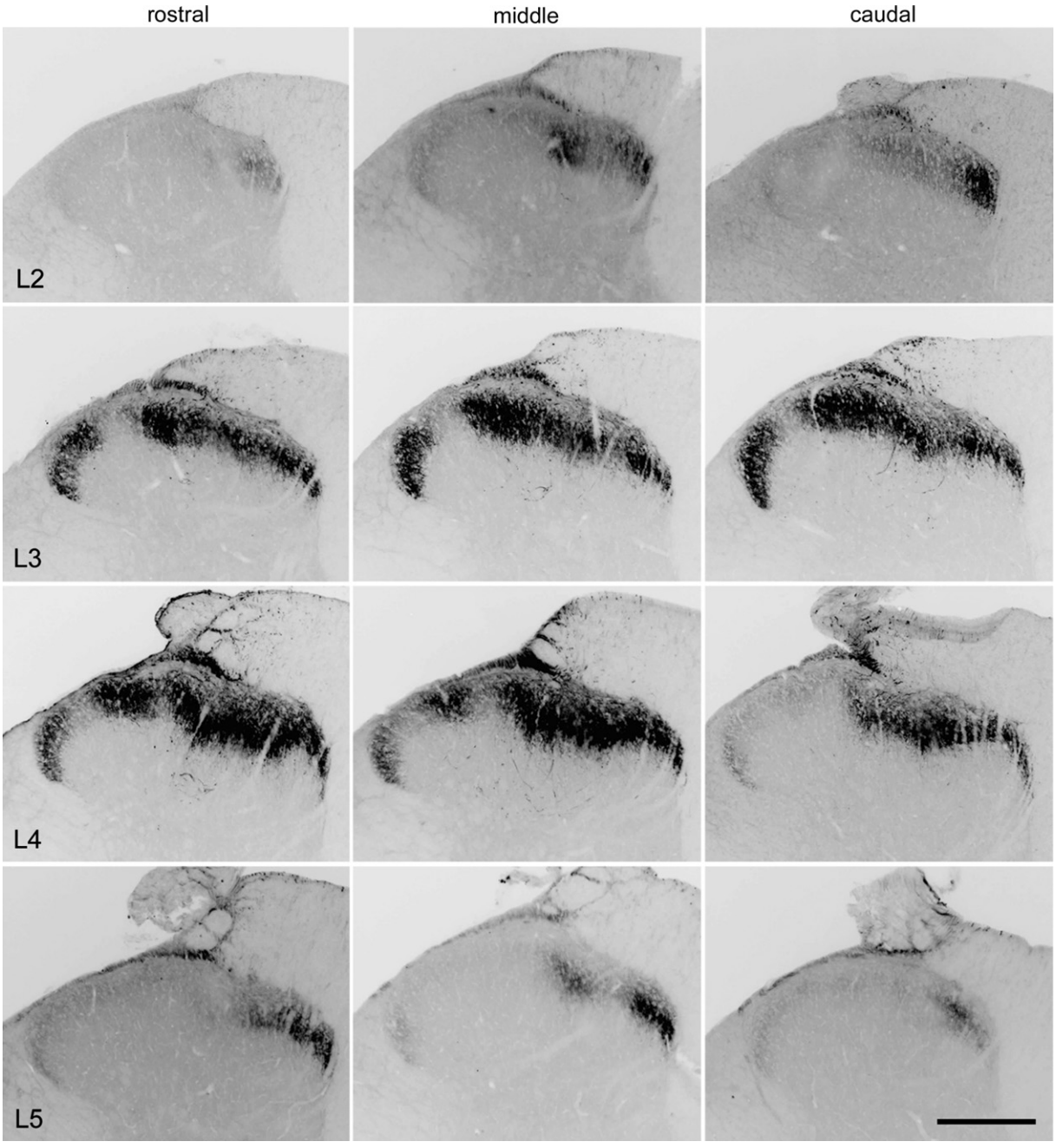

Fig. 1 – Transverse sections of the spinal cord showing IB4-labelled nerve terminals in the superficial dorsal horn at different rostrocaudal levels of lumbar segments following the injection of IB4 into L4 spinal nerves. Note the main nerve termination in the corresponding (L4) and one rostral (L3) spinal segment with less labelled nerve terminals in L2 and L5 segments. Scale bar = 0.25 mm.

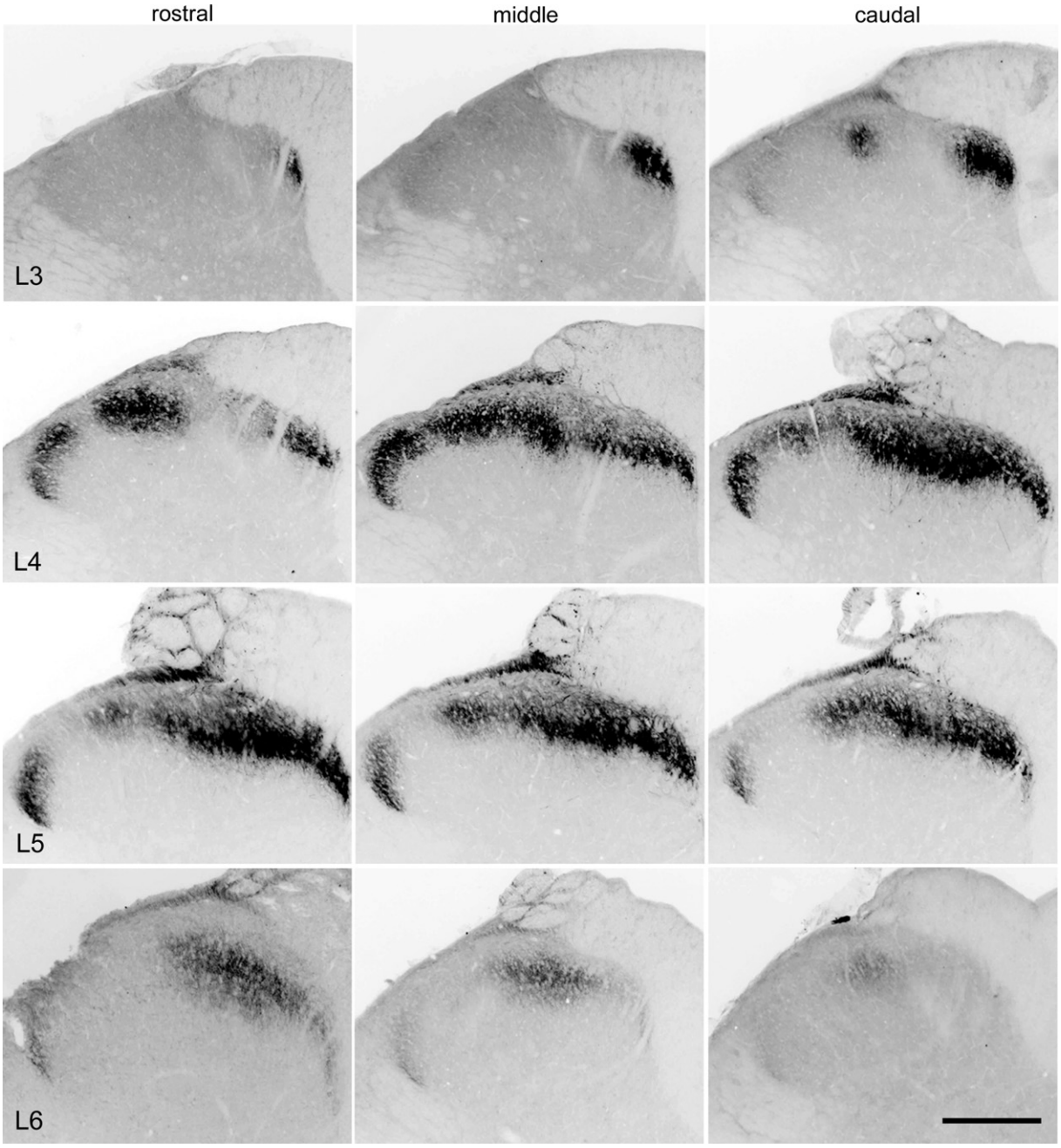

Fig. 2 – Transverse sections of the spinal cord showing IB4-labelled nerve terminals in the superficial dorsal horn at different rostrocaudal levels of lumbar segments following the injection of IB4 into L5 spinal nerves. Note the main nerve termination in the corresponding (L5) and one rostral (L4) spinal segment with less labelled nerve terminals in L3 and L6 segments. Scale bar = 0.25 mm.

the adjacent L4 segment where they could produce heterosynaptic sensitisation to input from L4 segment. This could account for the hyperalgesia to stimuli that is signaled by activity in the intact afferents". The aim of this study was, therefore, to provide anatomical evidence that might explain the mechanism of neuropathic pain following peripheral nerve injury. Specifically, we tested whether unmyelinated (C) fibres (many of which are nociceptive afferents) that belong to adjacent spinal nerves have overlapping territories within the dorsal horn of the spinal cord. If this proves to be the case, we hypothesise that it is likely that injury to one spinal nerve will lead to changes within a region of the dorsal horn that still receives input from adjacent (intact) nerves. These changes are likely to include loss of primary afferent-mediated inhibition, and this could explain why stimulation of an intact nerve adjacent to an injured nerve leads to the exaggerated sensations that are characteristic of neuropathic pain.

To test this hypothesis, two types of experiment were carried out to reveal the central termination of L5 and the fourth lumbar (L4) spinal nerves. In the first series of experiments

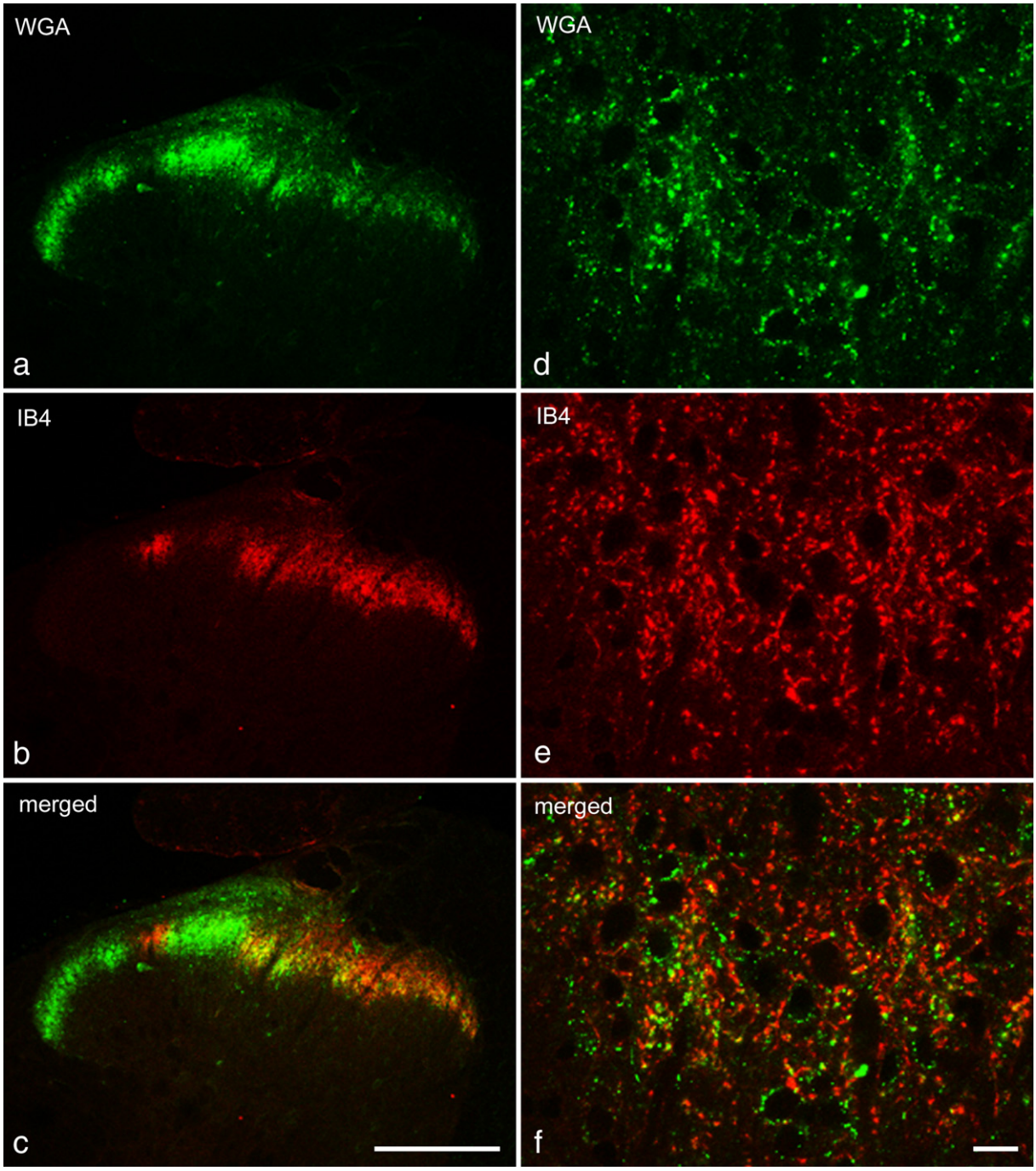

Fig. 3 – Confocal images of a transverse section of the L4 spinal cord segment showing the overlapping of WGA-labelled nerve terminals (green in a) and IB4-labelled nerve terminals (red in b) from a rat injected with IB4 in L4 and WGA in L5 spinal nerves. Note the overlapping of nerve terminations of L4 (red) and L5 (green) spinal nerves in the dorsal horn of L4 spinal segment. d and e are images of high magnification taken from the medial 1/2 of the dorsal horn showing overlapping but no colocalisation of labelled terminals. Panel c is a merged image of a and b and panel f is a merged image of d and e. Scale bar in c=250 μm and in f=10 μm.

rats received an injection of either wheat germ agglutinin conjugated to horseradish peroxidase (WGA–HRP) or *Bandeiraea simplicifolia* isolectin B4 (IB4) into the L4 or L5 spinal nerve. When WGA–HRP or IB4 are injected into peripheral somatic nerves, they are selectively taken up by unmyelinated afferents and transported transganglionically to the dorsal horn of the spinal cord, where they can be revealed with immunocytochemistry. In the second series of experiments, rats received injections of WGA into the L5 spinal nerve and IB4 into the L4 spinal nerve. Double-labelling immunofluorescence and confocal microscopy were used to reveal the two tracers in the dorsal horn with different fluorochromes. Some of the results of this study have been reported previously in abstract form (Shehab et al., 2007).

2. Results

2.1. Distribution of labelled WGA and IB4 terminals in the spinal cord

As previously reported (Robertson and Grant, 1985; LaMotte et al., 1991; Kitchener et al., 1994; Wang et al., 1994) injection of WGA–HRP, unconjugated WGA or IB4 into the nerves resulted in dense labelling mainly in lamina II of the spinal cord with invariably lighter labelling in lamina I. This is consistent with the view that C fibres terminate mainly in lamina II of the spinal cord (Sugiura et al., 1986; Sugiura et al., 1989; Willis and Coggeshall, 1991). In addition, a number of WGA-labelled motor neurons were found in the ventral horn in sections of the corresponding segment with only occasional IB4 labelled neurons in the entire corresponding segment were observed.

2.2. Distribution of unmyelinated primary afferents from the L4 spinal nerve

Injections of WGA–HRP or IB4 into the L4 spinal nerve resulted in labelling in the dorsal horn of L4 extended into two rostral spinal segments (L2 and L3) and one segment caudally (L5) with significant labelling in L3 and L4 (Fig. 1). In the caudal part of the L4 segment the plexus of labelled nerve terminals was mainly located in the medial half of the dorsal horn. The labelling extended to the lateral aspect of the dorsal horn in the rostral part L4 and in L3. The labelling in L2 was detected in a small area in the medial aspect of the dorsal horn. Caudally, a moderate to light density of labelled terminals was present in a small area in the medial half of the dorsal horn of L5. No labelling was detected in L1 or L6.

2.3. Distribution of unmyelinated primary afferents from the L5 spinal nerve in the dorsal horn of the spinal cord

Injection of WGA–HRP or IB4 into the L5 spinal nerve resulted in a pattern of labelling that was similar to that described above, but displaced caudally by one segment (Fig. 2). Labelled central terminals were observed in the dorsal horn of L5 and extended into two rostral spinal segments (L3 and L4) and one segment caudally (L6) with the densest labelling in L4 and L5. In the caudal part of the L5 the plexus of labelled nerve terminals was located mainly in the medial half of the dorsal horn. The labelling extended to the lateral aspect of the dorsal horn in the rostral part L5 and in L4. Labelling in L3 was detected in a small area restricted to the medial aspect of the dorsal horn. Caudally, moderate to light terminal labelling was detected in a small area in the medial half of dorsal horn of L6 (Fig. 2). No labelling was detected in L2 or S1.

2.4. Double immunofluorescent labelling and confocal microscopy

The results from single injections strongly suggest that there is considerable overlap between the central terminals of L4 and L5 spinal nerves within the dorsal horn. This was confirmed by examination of tissues from the rats that had received injections of IB4 into the L4 spinal nerve and WGA into the L5 spinal nerve. In these animals, double immunofluorescence labelling showed that there was an overlap of IB4- and WGA-labelled nerve terminals in the dorsal horn in L4 (Fig. 3a–c), L3 and L5 (not shown). Examination of the sections with the confocal laser microscope revealed that IB4-labelled and WGA-labelled boutons were intermingled in L4 with minimal colocalisation of the two markers (Fig. 3d–e). Quantitative analysis showed that only 3.8%±1.1 (means±SEM) of WGA-labelled terminals were also IB4 positively immunoreactive. The reasons for this low level of colocalisation are not clear and must be interpreted with caution. However, one possibility is that it results from extracellular labelling or transneuronal transport of WGA (Weinberg et al., 1990).

Labelling in all animals was detected only in the ipsilateral injected side of the spinal cord.

3. Discussion

The major findings of this study are twofold. First, unmyelinated primary afferents of the L4 and L5 spinal nerves were found to terminate mainly in the corresponding and next rostral spinal segments with some fibres ascending to terminate two segments rostral to their point of entry and some descending to terminate in the segment caudal to the level at which they entered. Secondly, double immunofluorescence labelling and confocal microscopy showed that the central terminations of unmyelinated primary afferents of the L4 and L5 spinal nerves intermingle in the dorsal horn at the L3–L5 levels. This overlapping of the primary afferents of two adjacent nerves might provide an explanation for the production of neuropathic pain following the peripheral nerve injury (see below).

3.1. Technical issues and central termination of unmyelinated primary afferents in the dorsal horn

In this study we have used two anatomical tracers, WGA (unconjugated or conjugated to HRP) and IB4, as markers for unmyelinated primary afferents. Our results confirmed previous work (Robertson and Grant, 1985; LaMotte et al., 1991; Kitchener et al., 1994; Wang et al., 1994) by showing that these tracers are transported to the superficial dorsal horn (particularly the ventral part of lamina II). This is also consistent with the known termination of C fibres in this region (Sugiura et al., 1986; Sugiura et al., 1989; Willis and Coggeshall, 1991) which are relatively selective for non-peptidergic afferents (Kitchener et al., 1994). Unfortunately, the lack of suitable transganglionic tracers for peptidergic C afferents makes it difficult to ascertain whether or not the peptidergic afferents show the same overlap of central arborisations in the dorsal horn of the spinal cord. However, previous data from our laboratory and others strongly suggest that both peptidergic and non-peptidergic afferents have a similar pattern of rostrocaudal and mediolateral termination in the dorsal horn of the spinal cord. For example, it has been demonstrated that peripheral nerve injury causes reduction in fluoride resistant acid phosphatase or IB4 binding (both of which are mainly associated with non-peptidergic afferents) in the superficial dorsal horn that has the same rostrocaudal and mediolateral distribution as the

downregulation of several neuropeptides (Substance P, Somatostatin, Cholecystokinin and CGRP) and upregulation of others (Vasoactive intestinal polypeptide and galanin, Shehab and Atkinson, 1986a,b; Shehab et al., 2003, 2004; Hokfelt et al., 1994, 1997, 2006).

3.2. *Mechanism(s) of production of neuropathic pain in spinal nerve ligation model*

The precise mechanisms that underlie neuropathic pain are not fully understood and there are many potential explanations (Yaksh and Sorkin, 2005; Devor, 2006), with a general agreement that it originates from a lesion in the nervous system (Campbell and Meyer, 2006). Although lesion of the central nervous system may lead to pain, most of the available models for neuropathic pain involve peripheral nerve injury (Bennett and Xie, 1988; Seltzer et al., 1990; Kim and Chung, 1992), with the SNL model of Kim and Chung (1992) being one of the most commonly used ones. There is debate about whether injured (Sheen and Chung, 1993; Han et al., 2000; Liu et al., 2000a,b) or uninjured (Ali et al., 1999; Li et al., 2000; Wu et al., 2001; Sheth et al., 2002; Shim et al., 2005; Djouhri et al., 2006; Jang et al., 2007) primary afferents are responsible for neuropathic pain (see also Gold (2000); Ringkamp and Meyer (2005); Campbell and Meyer (2006)), and also about the role of different classes of primary afferents (Ossipov et al., 1999; Liu et al., 2000a; Wu et al., 2001; Ringkamp and Meyer, 2005; Campbell and Meyer, 2006). For example, Li et al. (2000) showed that mechanical hyperalgesia after L5 ligation is not dependent on the injured afferents and proposed that adjacent uninjured nerve fibres contribute in the development and the maintenance of neuropathic pain. They also suggested that the peripheral degeneration of L5 axons may lead to hyperalgesia. However, this mechanism is unlikely to explain pain behavior that appears within one day of injury, since peripheral degeneration takes several days to develop (Li et al., 2000). Although the results of our study do not rule out the role of the peripheral interaction between the degenerating nerves and the intact nerve fibres, they strongly suggest that neuropathic pain depends on the contribution of uninjured adjacent primary afferent, but due to a central rather than a peripheral mechanism.

Studies have shown that spinal nerve sectioning results in central sensitisation of the neurons in the denervated region of the dorsal horn (Chapman et al., 1998; Ziegler et al., 1999; Ji et al., 2003). One of the probable causes of the central sensitisation after peripheral nerve injury might be a wide variety of physiological, neurochemical, neurotransmitter and receptor changes in the primary afferent neurons and the neurons of the dorsal horn of the spinal cord occurred (Shehab and Atkinson, 1986a,b; Goff et al., 1998; Hokfelt et al., 1994, 1997, 2006). One of these changes includes an upregulation of neurokinin 1 (NK-1) receptors (Abbadie et al., 1996; Goff et al., 1998; Malmberg and Basbaum, 1998; Hughes et al., 2007) which have been shown to be involved in regulating pain sensitivity and the production/maintenance of neuropathic pain (Nichols et al., 1999; Khasabov et al., 2002; Mantyh and Hunt, 2004; King et al., 2005). Sciatic nerve section in rats (Abbadie et al., 1996; Goff et al., 1998) and mice (Malmberg and Basbaum, 1998) and SNL of L5 in rats (Hughes et al., 2007) have been shown to cause NK-1 upregulation in the dorsal horn of the spinal cord where the primary afferents of the injured nerve terminate. Interestingly, NK-1 has also been shown to be upregulated in both L4 and L5 after L5 SNL (Hughes D. and Todd A. personal communication; our unpublished data). These findings fit well with the results of this study which showed that spinal nerve afferents do not only terminate in their corresponding spinal segment but extend to the rostral segments. In this case projection neurons in L4 with NK-1 upregulation, caused by L5 injury, would result in exaggerated responses to noxious or sub-noxious stimuli applied to the skin supplied by L4 nerve resulting in hyperalgesia.

An alternative mechanism contributing to hyperalgesia in SNL could be the loss of primary afferent input to inhibitory interneurons in regions of dorsal horn that contain both intact and axotomised C fibres. The potential role of loss of synaptic inhibitory transmission by GABA neurons in the dorsal horn has been considered to contribute to chronic pain (Scholz et al., 2005; Knabl et al., 2008). We suggest that projection neurons in L4 may receive inhibitory input from GABAergic neurons located in this segment that are innervated by C fibres in both L4 and L5 nerves. In this case, cutting the L5 nerve might reduce the inhibitory drive to the projection neuron. If the latter receives the majority of its excitatory input through the L4 nerve, this may lead to a relatively greater loss of inhibition, and thus an increase in the net excitatory drive to the cell, which could contribute to neuropathic symptoms.

4. Conclusion

The central terminations of the unmyelinated primary afferents of the L4 and L5 are not restricted to their corresponding spinal segments that they enter but extend to two spinal segments rostrally and one segment caudally. Together with results of the double-labelling experiment, the data of this study showed that the L4 and L5 spinal nerves have overlapping areas of termination in the L4 spinal segment. This suggests that some neurons in the region of overlapping are likely to receive synapses from afferents in both nerves. We are, therefore, proposing that in the spinal nerve ligation model of neuropathic pain, it is very likely that injured (L5) and uninjured (L4) nerves share, significantly, the same central termination at the same neurons in the dorsal horn of L4. In this case, the neurons located in regions of overlap may show exaggerated responses to activation of the intact (L4) nerve due to sensitisation resulting from injury to the adjacent (L5) nerve.

5. Experimental procedures

All experimental procedures were approved by the Animals Ethics Committee of the Faculty of Medicine and Health Sciences of the United Arab Emirates University and performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

5.1. *Surgical procedures and tracer injections*

Adult Wistar ($n=21$) of either sex (200–250 g) were anesthetized with a mixture of 73 mg/kg ketamine (100 mg/ml) and 7.4 mg/

kg xylazine (20 mg/ml) intraperitoneally. The skin of the back was longitudinally incised and the transverse process of fifth or six lumbar vertebra was excised to reveal the fourth or the fifth lumbar spinal nerve respectively.

In the first series of experiments, rats received injections of 1 μl of either WGA–HRP (2%, Vector) or IB4 (2%, Vector) into the L4 ($n=7$) or the L5 ($n=7$) spinal nerve on the left side through a fine glass micropipette that was inserted into the nerve. After the injections the area was washed with sterile normal saline. The muscles and the skin incisions were sutured in layers. After three days of recovery, the animals were deeply anesthetized with an overdose of urethane (1.5 ml, 25%) injected intraperitoneally and perfused with 4% freshly depolymerized formaldehyde or modified Zamboni's fixative (Shehab et al., 2003) in 0.1 M phosphate buffer (pH 7.4) through the ascending aorta. The spinal lumbar segments from L1 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 dissected out from those animals which had received IB4 injections and postfixed in the same fixative for another 2 h and then stored in 15% sucrose in phosphate buffer (pH 7.4) overnight.

To determine whether there was overlap of the arborisations of L4 and L5 primary afferents in the dorsal horn of the spinal cord we carried out another series of experiments in which rats ($n=7$) received injections of WGA (1 μl, 5%) into the left L5 spinal nerve and of IB4 (1 μl, 2%) into the left L4 spinal nerve. In a preliminary experiment we had found that following injection of IB4 into the L5 spinal nerve, some IB4 labelled neurons were present in the ipsilateral L4 DRG. This labelling was likely to have resulted from diffusion of the tracer from the L5 nerve to the L4 nerve following the injection, since the two join to form the sciatic nerve shortly after the L5 nerve leaves the intervertebral foramen. To prevent diffusion of tracers from one spinal nerve to another in the rats that received double injections, we therefore ligated and sectioned the L4 and L5 spinal nerves distal to injection site. The same procedure was used in rats which received single injection of IB4 in either L4 or L5 spinal nerve. Spinal cord segments L3–L5 and the L3–L6 DRGs on the left side were dissected out and stored as mentioned above.

5.2. *Immunocytochemistry*

5.2.1. *Immunoperoxidase and immunofluorescent stainings*

Transverse 50-μm thick sections of L1–L6 spinal segments were cut with a cryostat and collected serially. For each segment, sections from rostral, middle and caudal parts were then divided into 2 groups for immunoperoxidase or immunofluorescence staining. All sections were treated with 50% ethanol to increase antibody penetration (Llewellyn-Smith and Minson, 1992). For peroxidase staining, sections were incubated overnight either in biotinylated goat anti-WGA (1:10,000, Vector) or goat anti-IB4 (1:10,000, Vector). After rinsing, the sections were incubated with biotinylated donkey anti-goat (1:500, Jackson) for an hour followed by Extravidin peroxidase conjugate (1:1,000, Sigma). Finally, the sections were incubated for 5–10 min in a solution of 3,3′-diaminobenzidine containing hydrogen peroxidase and nickel chloride. All sections were mounted on gelled slides and allowed to air-dry overnight. They were then washed, dehydrated in graded alcohol, cleared in xylene, coverslipped and examined under the microscope. For immunofluorescent staining, sections were incubated with goat anti-IB4 (1:1,000) overnight. After washes in PBS, the sections were incubated with anti-goat IgG conjugated to Alexa 488 (Molecular Probes) and after further PBS washes they were mounted with glycerol–PBS medium. All the antibodies were diluted in PBS (pH 7.4) containing 0.3% Triton.

Cryostat sections (20 μm) of the L3–L6 DRGs were collected on gelled slide and stained with goat anti-IB4 or goat biotinylated anti-WGA antibodies using the peroxidase method as described above.

Photographs of stained sections were taken using an AxioCam HRc Digital camera with AxioVision 3.0 software to capture images (Carl Zeiss, Germany). The resulting files were used to generate the figures in Adobe PhotoShop (v. 7.0 Adobe Systems, Mountain View, CA).

5.3. *DRG*

In the first series of experiments, only sections of DRGs from animals that were injected with IB4 were stained and examined. Injection of IB4 in L4 spinal nerve resulted in positive IB4 immunoreactivity only in the L4 DRG with no labelling detected in L3, L5 or L6 DRGs. Similarly, in animals that received IB4 injection in the L5 nerve, IB4 immunoreactivity was only seen in the L5 DRG with no labelling detected in L3, L4 or L6 DRGs. In the second series of experiments, in which IB4 was injected into L4 and WGA into L5 spinal nerves, IB4 immunoreactivity was detected only in the L4 DRG, with no labelling in L3, L5 or L6 DRGs, while WGA immunoreactivity was only seen in L5 DRG, with no labelling detected in L3, L4 or L6 DRGs.

5.3.1. *Double immunofluorescent staining and confocal microscopy*

From those rats that received injections of WGA in the L5 spinal nerve and IB4 in the L4 spinal nerve, transverse sections (50 μm) from the rostral, middle and caudal parts of each segment were collected in different bottles and divided into 2 groups. For the first group of sections, double-labelling immunofluorescent method was employed to reveal both WGA and IB4 labelled terminals within the same section. The sections were incubated in a cocktail of primary antibodies: rabbit anti-WGA (1:15,000, Sigma) and goat anti-IB4 (1:1000). After rinsing, the sections were incubated for 2 h with a mixture of species-specific secondary antibodies (Donkey anti-rabbit IgG conjugated to Alexa 488, Molecular Probe, and donkey anti-goat IgG conjugated to Rhodamine Red, Jackson). Sections were mounted on glass slides with glycerol–saline medium and examined with Zeiss fluorescent microscope equipped with appropriate filters to reveal Alex 488 (green) and Rhodamine Red (red) or with a Bio-Rad MRC 1024 confocal laser scanning microscope (Hemel Hempstead, UK) equipped with a krypton–argon laser.

5.4. *Image analysis*

The results of the double-labelling experiment showed that WGA and IB4 labelled terminals were intermingled in the dorsal horn of the spinal cord. One section from each of five

rats was analysed to confirm that these different labels were not colocalised in the same terminals. Areas in the medial half of the dorsal horn were scanned with the confocal microscope through a 60× oil-immersion lens (1.5 zoom). For each field, twenty optical sections were scanned at 1 μm z-separation. Image stacks were then analysed with Photoshop software (version 7.0, Adobe Systems, Mountain View, CA, USA). Confocal stacks were initially viewed so that only the Alexa 488 (WGA) signal was visible, and 100 WGA-labelled boutons were randomly selected from each section from different stacks (separated at least 5 μm). The images corresponding to Alexa 488 and rhodamine were then viewed and the presence or absence of colocalisation of rhodamine (corresponding to IB4) was determined for each of the selected boutons.

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