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Simultaneous identification of unmyelinated and myelinated primary somatic afferents by co-injection of isolectin B4 and Cholera toxin subunit B into the sciatic nerve of the rat

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ABSTRACT

Several studies have used the transganglionic tracers cholera toxin subunit B (CTb) and either *Bandeiraea simplicifolia* isolectin B4 (IB4) or wheat-germ agglutinin (WGA) to label myelinated and unmyelinated afferent fibres respectively. In this study, we aim to determine whether co-injection of CTb and either IB4 or WGA into the sciatic nerve of rat will selectively label myelinated and unmyelinated simultaneously. A double immunofluorescence approach was used to detect these tracers in dorsal root ganglia (DRGs) and afferent fibre terminals in the spinal cord. CTb- and IB4-labelled neurons were seen mainly in L4 and L5 DRGs, with CTb labelling detected primarily in large sized neurons and IB4 staining seen mainly in smaller cells. Only a minority of CTb labelled DRG neuron profiles (5.1%) were also labelled with IB4. In the spinal cord, IB4-labelling was largely confined to lamina II of spinal segments L3–L5, whereas CTb-labelled terminals were seen in all laminae but sparse in lamina II. Confocal microscopy showed no evidence for colocalisation of CTb and IB4 labelling in any terminals in laminae I–III. Although the central distribution of CTb labelling in laminae I and II inner-IV had the same rostro-caudal and medio-lateral coverage as IB4 labelling in spinal segments L3–L5, CTb labelling in ventral laminae (of putative proprioceptor afferents) extended between T12 and S1. Similar patterns of central labelling were found when CTb and WGA were injected together. We therefore concluded that this co-injection approach provides a reliable method to identify both myelinated and unmyelinated somatic primary afferents simultaneously.

1. Introduction

Neuroanatomical tract-tracing methods have been widely used to study the neuronal connections between different parts of the nervous system. These powerful techniques allow us to study the origins, courses and patterns of termination of nerve fibres in detail. A number of neuroanatomical tracers have been used to study the central distribution of primary afferents in the spinal cord, including cholera toxin subunit B (CTb), wheat germ agglutinin (WGA) or *Bandeiraea simplicifolia* isolectin B4 (IB4). These tracers bind selectively to the GM1 ganglioside (CTb), N-acetyl-D-glucoseamine and N-acetylneuraminic acid (WGA) or terminal α-galactose (IB4) (Finkelstein et al., 1974; Trojanowski et al., 1982; Ganser et al., 1983; Scott et al., 1990; Goldstein and Winter, 1999) and are taken up by endocytotic mechanisms into myelinated (CTb, WGA), and unmyelinated (WGA, IB4) afferent fibres. In the dorsal root ganglia (DRGs), CTb is known to label mainly large and medium sized neurons with a mean profile area of 1060 μm^2 (Robertson et al., 1991), whereas most of the cells labelled with WGA and IB4 have mean profile areas with the peak at 500–600 μm^2 (Valtschanoff et al., 1992; Wang et al., 1994). In spinal cord sections, CTb-labelled terminals are found in laminae I and II inner (IIi) to IX, but are normally very sparse in the outer part of lamina II (IIo) (Robertson and Grant, 1985; LaMotte et al., 1991; Rivero-Melián and Grant, 1990, 1991; Woolf et al., 1992; Shehab et al., 2003, 2004; Shehab, 2009). In contrast, the central terminals of WGA- or IB4-labelled afferents terminate preferentially in lamina II (Swett and Woolf, 1985; Robertson and Grant, 1985; LaMotte et al., 1991; Kitchener et al., 1993,1994; Wang et al., 1994; Fullmer et al., 2004; Shehab et al., 2008; Shehab, 2009). While these studies illustrate that both myelinated and unmyelinated primary afferents can be labelled selectively using single injections of CTb, WGA or IB4, attempts to label both populations simultaneously with co-injections of CTb and WGA have been variable (LaMotte et al., 1991; Liu et al., 1995). Consequently, the primary aim of this study was to test whether myelinated and unmyelinated afferents could be labelled both selectively and simultaneously using a mixture of CTb and IB4 injected into the sciatic nerve. Similarly, we also aimed to verify whether a mixture of CTb and WGA could be used to label

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the myelinated and unmyelinated primary afferents respectively in the same animal. This technique will provide an invaluable approach for investigating the structural and spatial relationship between the central arbors of functionally distinct primary afferent fibres. For example, not only will this method allow us to determine whether myelinated and unmyelinated primary afferents from a particular nerve target the same or separate populations of dorsal horn neurons, but it will also allow us to investigate potential changes in afferent fibre labelling following peripheral nerve injury or inflammation. Such studies will further advance our understanding of the dorsal horn connectivity and the circuitry involved in processing both nociceptive and non-nociceptive afferent input.

2. Materials and methods

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.

2.1. Surgical procedures and sciatic nerve injection

Adult Wistar rats (body weight range: 210–244 g) were anaesthetised with a mixture of ketamine (80 mg/kg) and xylazine (16 mg/kg) delivered intraperitoneally. The skin of the back of the thigh was incised and the left sciatic nerve was exposed and injected with a mixture of 2 μl of either 1% CTb (Sigma–Aldrich, Taufkirchen, Germany) and 2% of IB4 (Vector Laboratories, Peterborough, UK, $n = 4$) or 1% CTb and 4% WGA (Vector, $n = 4$). In another three rats a mixture of 1% CTb and 2% WGA–HRP (Vector) was used. In order to have direct comparison between the effect of single and combined injections of these tracers, only CTb, IB4 or WGA was injected ($n = 3$ each) into the sciatic nerve. The tracers were injected into the sciatic at the mid-thigh level proximal to its major divisions into tibial, common peroneal and sural nerves. To ensure that the whole sciatic nerve was filled we used finely drawn glass micropipette inserted gently, for few millimetres, into the nerve in 3–4 different positions. In addition, the tracers were prepared in 0.1% solution of Fast Green. This allowed clear visualization of the injection process which resulted in colouration of all aspects of the nerve for approximately 1 cm length and also served to confirm that no leakage had taken place during, or immediately after, the injection. The muscles and the skin were sutured in layers. Three days after the injections of the tracers in all experiments, the animals were deeply anesthetized with an overdose of urethane (2 ml, 25%) injected intraperitoneally and perfused through the ascending aorta with modified 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 T12 to S1 were dissected out, post-fixed in the same fixative for 3–4 h, and stored in 30% sucrose in phosphate buffer overnight. L3–S1 dorsal root ganglia (DRGs) were also dissected out and post-fixed in the same fixative for another 1–2 h and then stored overnight in 15% sucrose in phosphate buffer (pH 7.4).

2.2. Immunocytochemistry

2.2.1. Spinal cord

Transverse sections (50 μm) of T12–S1 spinal segments were cut in a cryostat and treated with 50% ethanol to increase antibody penetration (Llewellyn-Smith and Minson, 1992) then incubated overnight in the following combinations of primary antibodies. From rats which were injected with CTb and IB4, the sections were incubated in a cocktail of mouse anti-CTb (a gift from M. Wikstrom, University of Goteborg, Sweden; diluted 1:50–1:100) and goat anti-IB4 (Vector; diluted1:1000). Some sections were also incubated with a mixture of rabbit anti-CTb (Sigma, diluted 1:2000) and goat anti-IB4 (Vector; diluted1:1000). From animals injected with CTb and WGA, sections were incubated in a mixture of mouse anti-CTb (diluted 1:50–1:100) and rabbit anti-WGA (Sigma, diluted 1:15,000). Sections were then incubated in a mixture of two species-specific secondary antibodies from anti-mouse, anti-rabbit and anti-goat IgGs conjugated to either Rhodamine Red (Jackson Immunoresearch; diluted 1:100) or Alexa 488 (Molecular Probes/Invitrogen; diluted 1:500) for 2 h. Sections were mounted on glass slides with glycerol-based anti-fade medium and examined using a Nikon Eclipse E600 fluorescent microscope equipped with appropriate filters to reveal Alex 488 and Rhodamine Red to determine the spinal levels of central labelling for each tracer. Representative digital images were captured using a Zeiss AxioCam HRc Digital camera with AxioVision 3.0 software (Carl Zeiss, Germany). Sections were also examined with either a Nikon C1 laser scanning confocal microscope or with a Bio-Rad Radiance 2100 confocal laser scanning microscope (Hemel Hempstead, UK) equipped with Argon, Green HeNe and Red diode lasers.

2.2.2. Dorsal root ganglia

L3, L4, L6 and S1 DRGs were sectioned in cryostat (20 μm) and mounted on gelatine subbed slides. Thick sections (60 μm) of L5 DRG were prepared and collected in small bottles and stained using floating method. All sections were incubated with a mixture of primary and secondary antibodies as described above to reveal CTb, IB4 and WGA labelling, then scanned with a Nikon confocal C1 laser microscope.

2.3. Image analysis

2.3.1. Spinal cord

Initial observations showed that CTb- and IB4-labelled terminals were localised in the superficial laminae of the dorsal horn of the spinal cord. Two sections from each rat ($n = 3$) were analysed further to determine whether these different labels were colocalised in the same terminals using a Bio-Rad MRC 1024 confocal laser scanning microscope. Areas from the medial half of the dorsal horn were scanned with a 60× oil-immersion lens. For each field, 11 optical sections were scanned at 0.5 μm z-separation. Image stacks were then analysed with Neurolucida for confocal software (MicroBrightFeild, Colchester, VT, USA). Briefly, the positions of 100 IB4-labelled boutons (selected at random) were first plotted on the confocal stacks when only the channel corresponding to IB4 labelling was visible. These stacks were then viewed for the channel corresponding to CTb to determine the frequency of CTb labelling in each of the selected IB4 boutons. The same protocol was used to randomly select 100 CTb-labelled boutons to determine the proportion of CTb-labelled terminals that also expressed IB4.

2.3.2. Dorsal root ganglia

Confocal images from at least two sections of L5 DRG per animal from each of the three rats were used to determine the frequency of CTb and IB4 colocalisation in DRG neurons. Adobe Photoshop software (version 7.0, Adobe Systems, Mountain View, CA, USA) was used to confirm the colocalisation of markers in individual cells, with only neuronal profiles showing obvious nuclei being included in the analysis. To determine the size of labelled CTb, IB4 and WGA immunoreactive profiles, the area of immunolabelled profiles for each marker was measured in L4 DRG from three rats. Four non-sequential sections (at least 60-μm apart) were analysed from each animal. Only profiles displaying obvious