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Lectin and Neuropeptide Labeling in Whole-Mount Preparation of Meninges in the Rat

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Abstract: The distribution of both peptidergic and non-peptidergic primary afferents in meninges was investigated in whole-mount preparations from meninges. Lectin *Griffonia simplicifolia* I-B4 (GSA I-B4) was used as a marker for non-peptidergic fibres. Calcitonin gene-related peptide (CGRP) was used as a marker for peptidergic fibers. Lectin *Griffonia simplicifolia* selectively labeled thin sensory axons that were distributed throughout meninges unrelated to blood vessels. CGRP immunoreactive axons, in contrast, were found to be very close to vascular patterns. This study demonstrates that lectin-positive fibres are constituted by a fibre population separate from those containing neuropeptides.

Key Words: lectin, calcitonin gene-related peptide, meninges, rat.

Beyin Zarlarının Whole-Mount Preparasyonlarında Lectin ve Neuropeptit Varlığının İncelenmesi

Özet: Beyin zarlarının whole-mount preparasyonlarında "peptidergic" ve "non-peptidergic" primer afferent sinir hücrelerinin dağılımı incelendi. Lectin *Griffonia simplicifolia* I-B4 (GSA I-B4) non-peptidegic sinir telleri için marker olarak kullanıldı. Calcitonin gene related peptide (CGRP) ise peptidergic olanlar için kullanıldı. Lectin pozitif ince sensorik neuronlarnı beyin zarlarında kan damarlarıdan bağımsız seyrettiği gözlendi. Buna karşın CGRP immunoreactiv axonların kan damarlarına bitişik olarak seyrettiği görüldü. Bu çalışma gösterir ki lectin pozitif sinir hücreleri neuropeptid içeren sinir hücrelerinden farklı bir populasyon içerir.

Anahtar Sözcükler: lectin, calcitonin gene-related peptide, beyin zarları, rat.

Introduction

Lectin *Griffonia simplicifolia* I-B4 (*Bandeiraea simplicifolia*) binds specifically to glycoconjugates that have a terminal α-D galactose (1,2). It has been demonstrated that GSA I-B4 selectively binds to a sub-population of small dorsal root ganglion neurons ("dark" or "Type B" sensory neurons), most of which are positive for the enzyme, fluoride-resistant acid phosphate (FRAP) (3). Most of FRAP-positive nerve fibres belong to the unmyelinated C-fibre population (3). From the distribution of FRAP in dorsal root ganglia, it has been proposed that FRAP-positive sensory neurons carry nociceptive information (4). Because of difficulties in determining the enzyme in peripheral terminals of the sensory fibres, probably due to low concentration in the terminals the lectin has been widely used instead. GSA I-B4 has been suggested as a useful marker for the "non-peptide" population of unmyelinated primary afferents (5), and has been clearly demonstrated in sensory ganglia (3, 5, 6, 7, 8, 9, 10), autonomic ganglia (7), the spinal cord (9, 11, 12) and the peripheral nervous system (3).

CGRP is a neuropeptide and usually a useful marker for the majority of peptidergic small-to medium-diameter "dark" or "Type B" sensory ganglion cells and fibres (3). Little co-localization has been observed between CGRP and GSA I-B4 in dorsal root ganglia (3, 5). It has been demonstrated that CGRP immunoreactive trigeminal neurons innervate cerebral meninges (13,14). With whole-mount preparation, CGRP-positive nerve terminals have been investigated in the central (13) and in the peripheral nervous system (3,15). We used whole-mount preparation of meninges to investigate the peripheral distribution of GSA I-B4- reactive nerve terminals in this region and to compare it with the distribution of CGRP immunoreactive fibres in meninges.

Materials and Methods

Experiments were performed on Wistar rats (of either sex). Animals were deeply anaesthetized with halothane (Fluothane) 4% in air and perfused transcardially with 100-200 ml Kreb's solution. The vascular rinse was followed by 300-500 ml fixation with 4% paraformaldehyde in 0.1M phosphate buffer saline (PBS) (pH 7.4). Crania were bisected along the medial sagittal

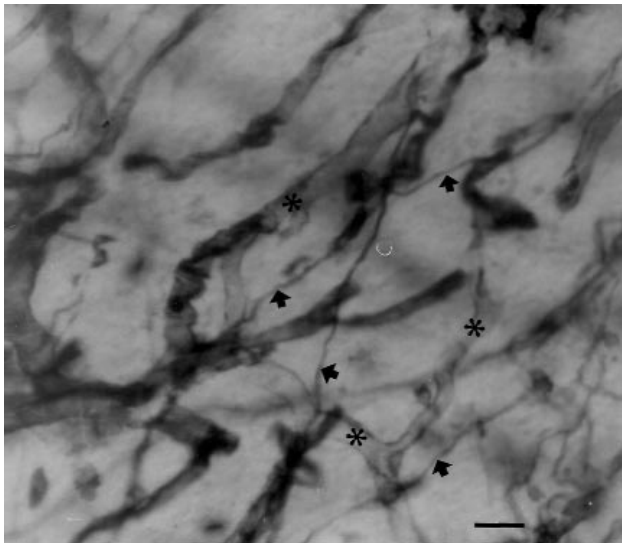

Figure 1. Meningeal whole-mount preparation of the rat. GSA ı-B4 labeled axons (arrows) traverse freely without relation to the blood vessels (stars) in the pia meter. Scale bar: 50 m

plane. After removal of the brain, pieces of cranial meninges which were constituted of dura mater, arachnoidea and pia mater, were individually dissected from the cranial cavity and brain. Meninges were stretched on clean slides and adhered by their edges. Then, tissues were postfixed with the same fixative overnight at 4˚C prior to immunohistochemistry.

Lectin histochemistry was performed with biotinilated GSA ı-B4 lectin (Sigma) as primary antibody. Whole mounts of meninges were washed for 1 h at room temperature in PBS. They were then incubated with lectin (5-10 mg/ml) diluted in PBS containing Triton X-100 and 2.5% bovin serum albumine overnight at room temparature. They were then incubated with streptavidin conjugated horseradish peroxidase (HRP), (1:100) (Amersham) for 1 h at room temperature. The chromogen protocol of Shu et al. (16) was used to reveal the distribution of HRP. Whole mounts were mounted onto chrome alum gelatine coded slides, dehydrated and coverslipped.

CGRP immunoreactivity was carried out with a set of protocol similar to those used for lectin histochemistry. The method was only modified for the biotinilation of the CGRP. In this case, after incubation with the CGRP (1:1000) (gift from Dr. P.K. Mulderry) whole-mounts were incubated with biotinilated anti-species antisera as the second step for 1-2 h at room temparature. In the control experiments, the lectin was pre-incubated with 0.1 M D-galactose (Sigma), which prevents the lectin staining. Absorption control was also used for the CGRP antibody.

Results

It was observed that the distribution of GSA I-B4-positive fibres contrasts with that of CGRP immunoreactive fibres in meninges. Lectin-positive axonal staining was distributed freely throughout the meninges and had no relation to blood vessels (Fig. 1), whereas CGRP immunoreactive nerve fibres were closely adherent to blood vessels (Fig.2a and 2b). Lectin-positive fibres were stained less intensely. CGRP immuroreactive axons could easily be traced as heavily labeled coarse nerve bundles. Nevertheless, some individual fine CGRP fibres were also distributed similarly to those of the lectins.

Discussion

The present study demonstrates the distribution of peptidergic and non-peptidergic primary afferent terminals in meninges, emphasizing that the lectin histochemistry can be used for visualization of a large number of undemonstrable FRAP-positive thin fibres (3). GSA I-B4 histochemistry has previously been used in whole-mount preparation of tunica vasculosa of testis and cornea (3). We used this preparation for meninges to investigate the lectin histochemistry. The preparation was found to be compatible with the lectin histochemistry, while its compatibility with peptides and particulary for CGRP has been confirmed (3,13,15,17)

It has been demonstrated that the soma, axon and central terminals of a subpopulation of small-diameter primary sensory neurons (C-fibres) are labelled by lectin soybean (18), which recognises the same population of cells bound with GSA I-B4 (5). Lectin has also been demonstrated in sensory neurons and their processes in the peripheral nervous system (3, 9). A possible developmental function has been implicated for lectins in sensory neurons (19). Hence, from its distribution in the central and periphery, it can be suggested that the GSA I-B4 reactive fibres observed in the present study participate in nociceptive mechanisms. However, a small number of unmyelinated autonomic axons has been reported (3). Therefore, the nociceptive function of the lectin-labeled neurons remains to be confirmed by further investigations, including electrophysiological studies of their receptive fields and identification of their biochemical structures in the meninges.

The close relationship found between CGRP fibres and blood vessels indicates that CGRP fibres participate in

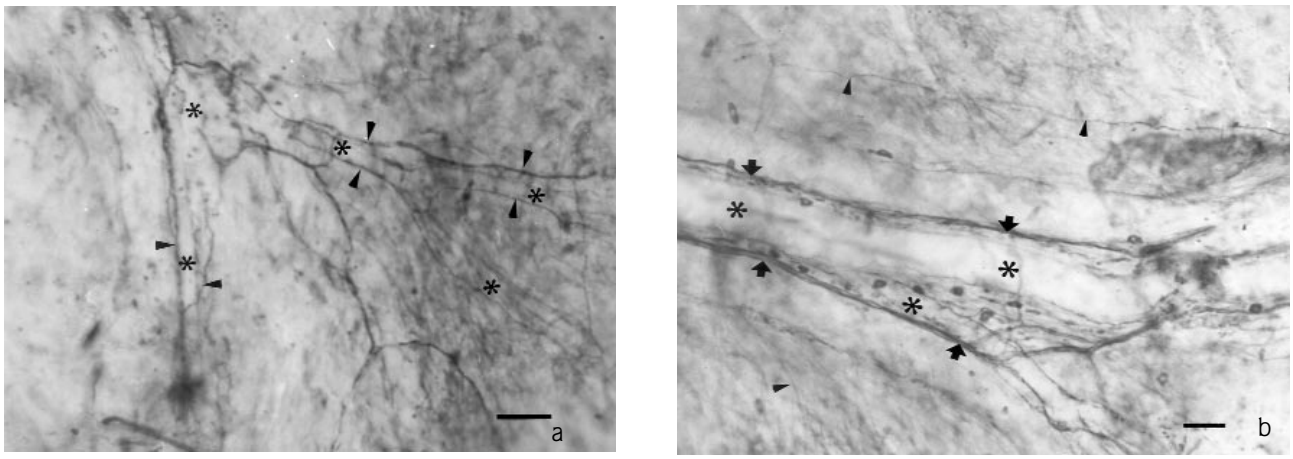

Figure 2. Whole-mount of dura mater reveals CGRP immunoreactive nerve fibres traversing (arrowheads) closely to the blood vessels (stars) (a). ınvestment of the vessel (stars) wall by these fibres (arrows) can be easily seen in a higher magnification (b). Some CGRP immunoreactive free fibres are also seen in the latter (arrowheads) . Scale bars: a: 50 µ, b: 20µ.

vascular innervation (20). The differences found in the innervation area of the markers suggest that the lectin histochemistry and CGRP immunohistochemistry label different fibre populations. The variety of fibre thicknesses observed between two population also supports this suggestion.

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