Available online at www.sciencedirect.com
SCIENCE DIRECT
Brain Research 1062 (2005) 86 – 91
BRAIN RESEARCH
www.elsevier.com/locate/brainres

Research Report

The majority of bladder sensory afferents to the rat lumbosacral spinal cord are both IB4- and CGRP-positive

Se Jin Hwang[a,b], Jung Min Oh[a], Juli G. Valtschanoff[b,*]

[a]Department of Anatomy and Cell Biology, College of Medicine, Hanyang University, Seoul 133-791, South Korea
[b]Department of Cell and Developmental Biology, CB #7090, University of North Carolina, Chapel Hill, NC 27599, USA

Accepted 25 September 2005
Available online 2 November 2005

Abstract

The rat urinary bladder is innervated by neurons in dorsal root ganglia (DRG) that express the neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP), and a fraction of bladder afferents can bind the non-peptidergic marker isolectin B4 (IB4). We used histochemical binding and axonal tracing to identify the bladder afferents, and immunocytochemistry to determine the degree of colocalization of CGRP with IB4 in their cell bodies in DRG and in their central axons in the spinal cord. In the L6 DRG, about 60% of CGRP-positive neurons were also positive for IB4. In the spinal cord, IB4 and CGRP colocalized in fibers and terminals in the inner part of lamina II, the lateral collateral path, and the sacral parasympathetic nucleus (SPN). In SPN, the majority of IB4-positive fibers and terminals were also CGRP-positive. After injection of IB4 into the bladder wall, immunoreaction for IB4 was detected in SPN, but not in lamina II. These results suggest that most IB4-positive afferents from the bladder are also CGRP-positive, and that the distinction between peptidergic and non-peptidergic bladder afferents based on IB4 binding is of limited validity.

Theme: Sensory systems
Topic: Somatic and visceral afferents

Keywords: Bladder; Sensory afferent; IB4; CGRP; Dorsal root ganglion; Neuropeptide

1. Introduction

The neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) are markers for nociceptive primary afferents [16]. SP/CGRP-containing dorsal root ganglion (DRG) neurons are referred to as peptidergic nociceptors and express the receptor for nerve growth factor, trkA [18]. Some small DRG neurons lack neuropeptides but can bind the isolectin B4 from *Bandeiraea simplicifolia* (IB4; [20,26]). These neurons may also mediate nociceptive stimuli; they express fluoride-resistant acid phosphatase (FRAP), cyclooxygenase [9,31], the receptor for ATP, $P2X_3$ [17], and depend on glial cell-derived neurotrophic factor (GDNF) for survival during postnatal development [18].

Central processes of IB4-positive neurons terminate predominantly in the inner part of lamina II (IIi) of the spinal dorsal horn, only partially overlapping with the peptidergic afferents, which terminate mainly in lamina I and the outer part of lamina II (IIo). IB4-positive afferents have also higher threshold and longer duration of action potentials, longer TTX-resistant currents, and smaller heat currents than IB4-negative afferents [27]. Moreover, the peptidergic afferents have been shown to sprout extensively in the spinal cord after dorsal rhizotomy, whereas IB4-positive neurons do not sprout and maintain their somatotopic organization [4].

Reflecting these differences, the commonly-used nomenclature divides the fine-caliber (C) primary afferents into (1) peptidergic expressing SP and/or CGRP, and (2) non-peptidergic binding IB4, notwithstanding the reports of IB4 binding by more than one-half of the SP/CGRP-positive

* Corresponding author. Fax: +1 919 966 1856.
E-mail address: jval@med.unc.edu (J.G. Valtschanoff).

0006-8993/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2005.09.026

afferents [6,14,28]. This dichotomy is further complicated by the heterogeneity of somatic nociceptors (cutaneous, muscular, articular) and by species differences. Furthermore, visceral nociceptors may differ from somatic nociceptors [8], yet investigators have focused onto cutaneous nociceptors mainly because they are more accessible to experimentation. For example, more is known about the role of SP in somatic pain than in visceral pain even though SP is expressed by a much higher proportion of visceral than cutaneous afferents (80% vs. 25%; [22]).

Lack of a clear attribution of afferents from somatic and visceral structures may hamper the interpretation of experimental data. For example, selective elimination of IB4-positive afferents to lamina II of the spinal dorsal horn was reported to suppress capsaicin-induced bladder overactivity [21], yet it is recognized that bladder afferents are predominantly peptidergic [5,32] and project to the sacral parasympathetic nucleus (SPN) and dorsal gray commissure (DGC; [19]), rather than to lamina II, and that the majority of peptidergic and much fewer non-peptidergic afferents are sensitive to capsaicin [27] or express the receptor for capsaicin TRPV1 [13].

We employ here histochemical binding and transganglionic tracing with IB4 from the urinary bladder combined with immunocytochemistry for SP and CGRP, and demonstrate that a large fraction of peptidergic bladder afferents are IB4-positive both at the level of their cell bodies in DRG and of their central terminals in the L6 segment of the spinal cord.

2. Materials and methods

Care and treatment of animals were according to the University of North Carolina IACUC guidelines. Male Sprague–Dawley rats (250–350 g) were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused with heparinized saline followed by 500 ml of 4% paraformaldehyde in phosphate buffer (PB, 0.1M, pH 7.4). L6–S1 spinal cord segments were removed, postfixed for 2 h in the fixative used for perfusion, and stored in cold PB. L6 DRG were removed and cryoprotected in 30% sucrose in PB overnight.

Forty micrometer-thick transverse sections of spinal cords, cut on a Vibratome, and of DRG, cut on a cryostat, were collected in cold PB. For immunofluorescent staining, sections were blocked with 10% normal donkey serum (NDS, Jackson) in phosphate-buffered saline (PBS, 0.01M, pH 7.2) for 10 min and incubated overnight with a mixture of rabbit anti-CGRP antibody (1:2,000, Immunostar) and guinea pig anti-SP antibody (1:2,000, Chemicon). Sections were rinsed and incubated in 2% NDS for 10 min and in a mixture of Cy3-conjugated donkey anti-guinea pig and Cy5-conjugated donkey anti-rabbit antibodies (1:200, Jackson) and IB4-FITC (1:100, Sigma) for 3 h. After several rinses, sections were coverslipped with Vectashield (Vector) and observed on a Leitz DMR microscope. Images were acquired with a cooled CCD camera (QImaging) and saved as TIFF files using OpenLab software (Improvision). Colocalization was examined with a Leica SP2 confocal microscope (Leica); triple fluorescent images were saved in TIFF format and contrast and brightness were adjusted with Photoshop v. 7 (Adobe).

For quantitative evaluation of double and triple labeled DRG neurons, we obtained data from 6 to 7 sections from two L6 DRG from each of four rats. In each section, we acquired one image (430 × 330 μm) with a CCD camera (F-View, SIS) using the same shutter speed and digital gain. Double- and triple-labeled cells were counted using AnalySIS software (SIS), and statistics (ANOVA, to check for variability between animals, significance was set at $P < 0.05$) were performed with Excel 2003 (Microsoft).

For neuroanatomical tracing of bladder afferents, 6 rats were anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (8 mg/kg) and the urinary bladder was exposed through median laparotomy. We used Fast Blue (FB, four rats) to label all bladder afferents, and IB4 (two rats) to label IB4-binding afferents. One percent FB or 1% IB4 (in 5% aqueous solution of dimethyl sulfoxide, 10 injections of 0.2 μl each) were injected into the wall of the urinary bladder through a 35-G needle attached with thin polyethylene tubing to a Hamilton syringe. After 3 days, rats were sacrificed with perfusion. DRG and spinal cord sections were processed for immunocytochemistry as above; FB fluoresces at 430 nm and is detectable under ultraviolet illumination; to visualize IB4 and CGRP, we used goat anti-IB4 (1:2000, Vector) and rabbit anti-CGRP (1:1000, Immunostar) primary antibodies and FITC-conjugated donkey anti-goat and Cy5-conjugated donkey anti-rabbit secondary antibodies (1:200, Jackson). Quantitative evaluation of double and triple-labeled neurons and statistics were performed on 7–8 DRG sections of each of four rats as above.

The antibodies used in this study are well characterized and are routinely used in our laboratory [2,12]: both the rabbit anti-CGRP and the guinea pig anti-SP antibodies show specific staining in rat tissues and immunostaining is completely abolished by preadsorption with the immunizing peptides. Additionally, we processed sections as above, except that primary or secondary antibodies were omitted. Omission of antibodies eliminated specific staining. To determine boundaries of spinal laminae, we used images acquired with DIC optics on the fourth channel of the confocal microscope.

3. Results

The majority of DRG neurons labeled with either SP or CGRP were also positive for IB4 (Fig. 1A). Out of 1711 neurons that labeled for CGRP in 29 sections from L6 DRG of four rats, 998 (58 ± 2%) were also positive for IB4, and out of 2468 neurons that labeled for IB4, 998 (40 ± 1%)