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Research article

Direct communication of the spinal subarachnoid space with the rat dorsal root ganglia

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ABSTRACT

The anatomical position of the subarachnoid space (SAS) in relation to dorsal root ganglia (DRG) and penetration of tracer from the SAS into DRG were investigated. We used intrathecal injection of methylene blue to visualize the anatomical position of the SAS in relation to DRG and immunostaining of dipeptidyl peptidase IV (DPP-IV) for detecting arachnoid limiting the SAS. Intrathecal administration of fluorescent-conjugated dextran (fluoro-emerald; FE) was used to demonstrate direct communication between the SAS and DRG. Intrathecal injection of methylene blue and DPP-IV immunostaining revealed that SAS delimited by the arachnoid was extended up to the capsule of DRG in a fold-like recess that may reach approximately half of the DRG length. The arachnoid was found in direct contact to the neuronal body-rich area in the angle between dorsal root and DRG as well as between spinal nerve roots at DRG. Particles of FE were found in the cells of DRG capsule, satellite glial cells, interstitial space, as well as in small and medium-sized neurons after intrathecal injection. Penetration of FE from the SAS into the DRG induced an immune reaction expressed by colocalization of FE and immunofluorescence indicating antigen-presenting cells (MHC-II+), activated (ED1+) and resident (ED2+) macrophages, and activation of satellite glial cells (GFAP+). Penetration of lumbar-injected FE into the cervical DRG was greater than that into the lumbar DRG after intrathecal injection of FE into the cisterna magna. Our results demonstrate direct communication between DRG and cerebrospinal fluid in the SAS that can create another pathway for possible propagation of inflammatory and signaling molecules from DRG primary affected by peripheral nerve injury into DRG of remote spinal segments.

1. Introduction

The subarachnoid space (SAS) filled by cerebrospinal fluid (CSF) is much discussed clinically in the field of chronic pain. It is a common site for application of anesthetics (Czernicki et al., 2014) and sampling of CSF when assessing chronic pain (Zin et al., 2010). Moreover, neuroinflammatory reaction expressed by upregulation of cytokines (TNFa, IL-6 and IL-10) and chemokines (SDF1/CXCL12) has been detected in dorsal root ganglia (DRG) both associated and non-associated with damaged sciatic nerve. These findings suggest that signaling of the molecular changes might be propagated alongside the neuraxis from affected to remote DRG (Jancalek et al., 2010, 2011; Dubový et al., 2010, 2013). Moreover, various types of nerve injury-induced neuroinflammatory reactions have also been found in the central nervous system structures (Vania Apkarian et al., 2006). We hypothesize that direct communication between the SAS and DRG could provide a pathway for propagation of signaling molecules alongside the neuraxis among DRG of different spinal segments via CSF. The first goal of our study, therefore, was to visualize the morphological position of the SAS in relation to rat DRG. Second, we investigated the penetration of 10 kDa fluorescent-conjugated dextran (fluoro-emerald; FE) from the SAS into DRG.

2. Materials and methods

2.1. Animals and surgical procedures

The experiments were performed on 20 adult male rats (Wistar, 200–250 g; Animal Breeding Facility of Masaryk University, Czech Republic). The animals were kept at 22 °C and maintained on a 12 h light/dark cycle under specific pathogen-free conditions in the animal housing area of Masaryk University. Sterilized food and water were available *ad libitum*. All experimental procedures were carried out aseptically and according to protocols approved by the Ethical Committee of Masaryk University, Brno and the Departmental

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2.2. Anatomical study of the SAS in relation to DRG

2.2.1. Intrathecal methylene blue injection

Although fusion of the meninges with DRG has been described (Shanthaveerappa and Bourne, 1962; McCabe and Low, 1969), a position of SAS in relation to rat DRG remained unclear. Therefore, we injected Methylene blue (MB) into SAS to macroscopically visualize the precise position of SAS in relation to DRG. Animals ($n = 5$) were anesthetized using a mixture of ketamine (40 mg/ml) and xylazine (4 mg/ml) administered intraperitoneally (0.2 ml/100 g body weight). MB was dissolved in artificial cerebrospinal fluid (ACSF; Hylden and Wilcox, 1980) at 1% concentration and 50 μl of this solution was injected into the SAS of the cisterna magna. The micro syringe was inserted through the posterior atlanto-occipital membrane after skin incision and blunt reflection of paravertebral muscles. Animals were sacrificed in CO_2 and perfused transcardially with 4% paraformaldehyde. Lumbar (L4–L5) and cervical (C7–C8) DRG were detected *in situ* following laminectomy and foraminotomy conducted with great care not to damage the meninges. Microdissection and analysis were performed under a stereomicroscope (Leica M50 LED) equipped with a digital camera (Leica DFC 480, Leica Microsystems, Wetzlar, Germany).

2.2.2. Detection of the arachnoid by DPP-IV immunostaining

Five rats were sacrificed in CO_2 and perfused transcardially with Zamboni's fixative solution (Zamboni and de Martino, 1967). DRG of cervical (C7–C8) and lumbar (L4–L5) segments were removed and then immersed in Zamboni's fixative overnight. The DRG were then washed in 20% phosphate-buffered sucrose (pH 7.2) and embedded in Tissue-Tek OCT compound (Miles; Elkhart, IN, USA). Longitudinal cryostat sections (12 μm) were then cut (Leica 1800 cryostat) and collected on gelatin-coated microscope slides. The sections were processed for indirect immunofluorescence staining to detect DPP-IV as a marker of the arachnoid (Haninec and Grim, 1990). Affinity purified secondary antibody conjugated with FITC was used for visualization of DPP-IV immunostaining (Table 1). Cell nuclei were stained with Hoechst 33342 (Sigma; St. Louis, MO, USA), the sections were mounted in a Vectashield aqueous mounting medium (Vector Laboratories; Burlingame, CA, USA) and analyzed using a Nikon Eclipse NI-E epifluorescence microscope equipped with a Nikon DS-Ri1 camera operated by a NIS-Elements software (Nikon, Prague, Czech Republic).

2.3. Penetration of labeled dextran (fluoro-emerald) from the SAS into DRG and immunodetection of loaded cells

Ten rats were deeply anesthetized using a mixture of ketamine (40 mg/ml) and xylazine (4 mg/ml) administered intraperitoneally (0.2 ml/100 g body weight). Fluorescent-conjugated dextran (fluoro-emerald, FE, MW = 10 kDa, lysine fixable, Molecular Probes, Eugene, OR, USA) was dissolved in ACSF (1.5 mg in 100 μl). The solution (50 μl) was injected into the SAS using the micro syringe through the atlanto-occipital membrane to the cisterna magna (cervical, $n = 5$) or between the L2 and L3 vertebrae (lumbar, $n = 5$). All rats were left to survive for 20 h. They were then sacrificed in CO_2 and perfused transcardially with Zamboni's fixative solution (Zamboni and de Martino, 1967). Cervical (C7-C8) and lumbar (L4-L5) DRG were removed, immersed in Zamboni's fixative overnight. The samples were then washed in 20% phosphate-buffered sucrose for 12 h and longitudinal cryostat sections (12 μm) were prepared. A part of the sections was stained using Hoechst 33342 (Sigma; St. Louis, MO, USA), mounted in a Vectashield aqueous mounting medium (Vector Laboratories; Burlingame, CA, USA), then analyzed using a Nikon epifluorescence microscope to examine FE penetration.

2.3.1. Immunofluorescence staining

To identify FE-loaded cells or cell reaction following FE penetration into DRG, the sections were processed for standard indirect immunofluorescence staining with ED1 and ED2 antibodies to detect activated and resident macrophages, respectively. In addition, MHC-II antibody was used to detect antigen-presenting cells (APC), GFAP antibody was applied as a marker of activated satellite glial cells (SGC), and Iba1 antibody was used to detect phagocytic activity of DRG cells. In addition, Ki-67 immunostaining was used to visualize proliferation activity of the cells. Primary and secondary antibodies and their concentrations are listed in Table 1. Briefly, the sections were washed with phosphate-buffered saline (PBS) containing 0.3% bovine serum albumin (BSA) and 0.1% Tween-20; treated with 3% normal goat or donkey serum for 30 min; then incubated with primary antibody at room temperature for an appropriate time and affinity purified secondary antibodies conjugated with TRITC were applied at room temperature for 90 min. The sections were washed in PBS, stained with Hoechst 33342, mounted in a Vectashield aqueous mounting medium, and then analyzed using a Nikon epifluorescence microscope. The control sections were incubated while omitting the primary antibodies.

2.3.2. Double immunostaining for GFAP and ED1

A part of DRG sections was double immunostained for GFAP and ED1 to detect positions of activated macrophages in relation to sensory neuron-SGC units. Sections were treated with a mixture of acetone and methanol (1:1) at −20 °C for 5 min, washed with PBS containing 0.3% BSA and 0.1% Tween 20, then treated with 3% normal goat serum for 30 min. The sections were then incubated with mouse monoclonal anti-ED1 antibody at room temperature and TRITC-conjugated goat anti-mouse secondary antibody for 90 min. Next, simultaneous immunostaining was done by incubating the sections with rabbit polyclonal anti-GFAP antibody at room temperature. After thorough washing in PBS, the sections were treated with AlexaFluor 350-conjugated goat anti-rabbit secondary antibody for 90 min. Sections were mounted in a Vectashield aqueous mounting medium (Vector Laboratories; Burlingame, CA, USA) after washing in redistilled water.

Table 1
Primary and secondary antibodies, their dilutions and producers.

Primary antibody	Type of antibody	Dilution; incubation time	Producer	Secondary antibody	Dilution	Producer
DPP-IV	Mouse monoclonal	1:200; 240 min	Santa Cruz, USA	Goat anti-mouse	1:100	Chemicon
ED1	Mouse monoclonal	1:200; 240 min	Serotec, UK	Goat anti-mouse	1:50	Chemicon
ED2	Mouse monoclonal	1:200; 16 h	Serotec, UK	Goat anti-mouse	1:100	Chemicon
GFAP	Rabbit polyclonal	1:250; 180 min	Dako, Denmark	Goat anti-rabbit	1:100	Chemicon
				Donkey anti-rabbit	1:400	Jackson ImmunoResearch
Iba1	Goat polyclonal	1:50; 16 h	Abcam, UK	Biotinylated Donkey anti-goat	1:400	Santa Cruz
Ki-67	Rabbit polyclonal	1:500; 240 min	Vector, USA	Goat anti-rabbit	1:100	Chemicon
MHC-II	Mouse monoclonal	1:100; 240 min	Serotec, UK	Goat anti-mouse	1:100	Chemicon