NEUROKININ 1 RECEPTOR, IB\textsubscript{4} LECTIN AND NITRIC OXIDE SYNTHASE LOCALIZATIONS IN WHOLE-MOUNT PREPARATION OF THE RAT MENINGES FOLLOWING NOXIOUS STIMULATION

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Distribution and immunocytochemical characterization of the nerve fibers and their terminals in Wistar rats \((n=15)\) meninges were studied by using neurokinin 1 receptor, IB\textsubscript{4} lectin and nitric oxide synthase labeling after the application of noxious peripheral stimulation. For immunocytochemistry, free floating specimens were incubated with neurokinin 1 receptor, nitric oxide synthase and IB\textsubscript{4} lectin antibodies and then observed with avidin-biotin-peroxidase method. The neuronal markers stained in all unmyelinated nerve fibres throughout the meninges. Neurokinin 1 receptor- and IB\textsubscript{4} lectin-immunoreactive nerve fibers were localized in all leptomeningeal compartments, where they terminated close to the subarachnoid space or within the connective tissue, thus suggesting extensive distribution of nerve fibres in the meninges. Neuronal nitric oxide synthase-positive nerve fibers and a prominent mast cell population were seen in the rat leptomeninx and dura. The presence of a significant number of putative nociceptive fibres suggests a possible role for nociceptive function in the context of pathophysiological aspects. The distributions pattern of IB\textsubscript{4} lectin binding sites, neurokinin 1 receptor and nitric oxide synthase could be due to the function of particular segments in the pathogenesis of meningeal vascular network.

Key words: neuronal markers, immunohistochemistry, pain, distribution, brain meninges

INTRODUCTION

The brain and spinal cord are covered by the layer of supporting tissues collectively called the meninges. The meninges are subdivided into the pachymeninx (dura mater) and the leptomeninx (arachnoid and pia mater). A distinct border of neurothelial cells separates the dura mater from the outer arachnoid cell layer. The space between the pia and arachnoid layers is called subarachnoid space. This space is lined by flattened mesothelial cells (Burkitt et al., 1993). Meninges are divided into: the superior sagittal sinus, the inferior
sagittal sinus, the straight sinus, the transverse sinuses, the confluence of the sinuses, and the antero-inferior sinuses (Greene, 1963).

The transmission and modulation of the pain system has a complex network. Peripheral nerve endings surround meningeal vessels (trigeminovascular system) and contain neuropeptides such as substance P (SP) and neurokinin A (NKA). Cephalic pain transmission takes place via activation of the trigeminovascular system, constituted by meningeal and superficial cortical blood vessels that contain sensory fibres (Mitsikos and Sanchez, 2001). The high-intensity stimulation of the dura mater along the blood vessels elicited pain in man, whereas stimulation of the connective tissue of the dura mater did not result in pain (Ray and Wolff, 1940).

Specific to headache pathophysiology is the activation of C-fibers leading to transmission of nociceptive information into the brainstem, chiefly the trigeminal nucleus caudalis and promotion of a sterile inflammatory response within the target tissues (Mitsikostas and Sanchez, 2001). In experimental models, chemical, mechanical or electrical activation of the C- and Aδ-fibres of the trigeminal nerve induce c-fos expression within specific areas of the trigeminal nucleus caudalis via release of neurotransmitters from the central terminal (Bereiter and Benetti, 1996). Many small sensory fibres in their peripheral terminal contain and release peptides from the perivascular endings (SP, calcitonin gene–related peptide–CGRP– and neurokinin) generating a sterile neurogenic inflammation (Edvinsson et al., 1983). SP acts on the neurokinin 1 (NK1) receptor on endothelial cells of postcapillary venules to induce gap formation, plasma protein extravasation and promote adhesion and infiltration of neutrophils (Bowden et al., 1994). The neurogenic inflammation theory of migraine pain proposes that SP, acting through NK1 receptors, causes dural inflammation, which enhances migraine pain.

Injection of lipopolysaccharides intraperitoneally increases the capsaicin-induced c-fos Li within trigeminal nucleus caudalis, through the generation of nitric oxide (NO) with subsequent activation of C-fibres and release of CGRP (Kemper et al., 1998). Stimulation of the superior sagittal sinus electrically or mechanically (Kaube et al., 1992) induces the release of neuropeptides (CGRP, but not SP or neuropeptide Y- NPY) in a pattern similar to that observed during migraine attacks (Edvinsson and Goadsby, 1994).

The study of Andres et al. (1987a) revealed that the majority of nerve fibres innervating the dura mater encephali in rats terminated in two locations as free nerve terminals. Firstly, at various segments of the meningeal vessels and venous sinuses and secondly, in the connective tissue. They also demonstrated mechanoreceptive and most likely nociceptive afferents at different segments of the meningeal vessels and in the connective tissue of the dura mater. The existence of nerve fibres supplying the mammalian cerebral arteries was further demonstrated by other morphological studies (Nelson and Rennels 1970; Nielsen et al., 1971). Different types of cerebrovascular nerve fibres containing neuropeptides and neurotransmitters such as SP and nitric oxide (NO) supply the cerebral arteries (Liu-Chen et al., 1986; Saito et al., 1987; During et al., 1990; Estrada, 1993; Shimizu, 1994). A number of studies showed that α-D-galactose
(αGal) binding lectins, such as the IB₄ lectin from Bandeiraea simplicifolia (BSI-B₄), binded to the cell bodies, axons and synaptic terminals of unmyelinated cutaneous primary sensory afferents (Plenderleith et al., 1990; Cameron et al., 1991; Kitchener et al., 1994).

The organisation and cellular composition of the connective tissue building up the microenvironment of the nerve fibre terminals play a crucial role in the physiological and pathophysiological mechanisms. Immunocytochemical characteristics of the nerve fibres in the leptomeningeal are still lacking. Therefore, the aim of this work was to determine the distribution of peptidergic nerve fibres and their terminals in the leptomeningeal and dural compartments of Wistar rats.

MATERIALS AND METHODS

Fifteen adult rats (Wistar) of either sex weighing approximately 250 g, were anaesthetised with urethane (1 mg/kg, i.p.). Mustard oil (Allylisothiocyanate, Merck) (40% in liquid paraffin) prior to vascular perfusion was applied four times with a paintbrush to the dorsal and plantar surfaces of the right hind paw up to the ankle joint, at 30 minutes intervals. Two hours after the first noxious stimulus, the animals were given a lethal dose of pentobarbitone (60 mg/kg, i.p.) and fixed by vascular perfusion with oxygenated Kreb's solution in which heparin (1000 IU/L) was added to remove the blood from the circulation, followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) (pH 7.2). The tissue blocks were prepared. They were then postfixed in the same fixative 4-6 hours and then cryoprotected with 30% sucrose in 0.1 M PBS overnight at 4°C. For immunocytochemistry, whole-mount preparation of the meninges were collected into tubes containing 0.1 M PBS and processed as free-floating specimens during all incubation steps. The endogenous peroxidase and non specific binding sites for antibodies were suppressed by treating sections with 1% hydrogen peroxide for 30 minutes and 10% normal donkey serum for an hour at room temperature, respectively. Sections were further processed for standard immunocytochemistry by the avidin-biotin-peroxidase complex (ABC) technique (Hsu et al., 1981).

The sections were incubated with primary antibodies to anti-neurokinin 1 (NK1) receptor raised in rabbits (1:20,000, kindly supplied by Dr. S. Vigna, Duke University Medical Center, NC, USA), neuronal nitric oxide synthase (nNOS) (1:5000, a kind gift from Dr. P. Emson, The Babraham Institute, Cambridge, UK) raised in sheep and IB₄ lectin in PBS containing 2.5 % normal donkey serum, 2% tritonX-100 and 0.25 % sodium asde, at room temperature for 72 hours and 4°C, respectively. Subsequently, the distribution of primary antiserum was detected using biotinylated anti-species secondary antibodies (biotinylated anti-rabbit (1:200; Amersham International plc. Buckinghamshire, UK); biotinylated anti-sheep (1:1000, Jackson Immuno-Research Lab., West Grove, PA), for an hour at room temperature and streptavidin-conjugated horseradish peroxidase (1:1000) (Amersham). The distribution of bound peroxidase was visualised using a reaction mixture containing 0.05 % 3',3'-diaminobenzadine HCl (DAB) with nickel (2.5% nickel ammonium sulphate) in sodium acetate buffer (pH 6.0) with 0.2% D-
glucose, 0.04% ammonium chloride and 0.1% glucose oxidase to supply oxygen (Shu et al., 1988). The sections were monitored under light microscope.

RESULTS

NK1 receptor-immunoreactive (IR) fibres were observed throughout the meninges. A remarkable network of NK1 receptor-like immunoreactive (LI) nerve fibres was located in the walls of the sagittal and transverse sinuses (Figure 1). NK1 receptor-LI nerve fibres were rare in the cerebral meninges (Figure 1). Their nerve fibers usually observed in the adventitial leptomeninx close to the basilar and anterior cerebral artery. The parietal dura mater covering the olfactory bulb and ventral dura was supplied by a moderate number of NK1 receptor positive nerve fibres. They run towards the sagittal sinus independent from vascular structures (Figure 1).

Our immunohistochemical study using neuronal nitric oxide synthase (NOS) antibodies revealed NOS-positive nerve fibers and a prominent mast cell population in the rat leptomeninx and dura (Figures 2 and 3). A majority of the immunopositive NOS-IR nerve fibers were associated with the adventitial meninges of the main cerebral arteries and its branches (Figures 2 and 3). A few thick fibre bundles primarily followed the long axis of the vessels, but finally supplied with a dense plexus of fine beaded axons oriented mostly circularly to the axis of the vessels (Figures 2 and 3).

Immunoreactivity for IB₄ lectin was observed within nerve fibres in the walls of all rat examined cerebral arteries (Figure 4). IB₄ lectin immunoreactive nerve fibres were distributed in the adventitial meninges of the cerebral arteries (Figure 4). Fibres coursed in all directions in relation to the long axis of the blood vessels.
Figure 2. NOS containing immunoreactive nerve fibres plexus in the dura mater. Note that axonal branching and dense network of fibres coursing to the long axis of the vessel. Bar: 50 μm

Figure 3. Nerve fibres plexus of the leptomeninx immunostained for the neural marker NOS. Bar: 100 μm

Figure 4. IB₄ lectin-LI nerve fibre plexus in close association with the branches of the meningeal artery and within the adjacent leptomeningeal artery. Bar: 100 μm
DISCUSSION

In the current study, the presence of a substantial number of nerve fibres immunoreactive for NK1 receptor, NOS and IB₄ lectin has been detected in all leptomeningeal compartments of the rat. Morphological data dealt with the innervation of the leptomeninges mainly focused on the cerebral arteries in whole mount preparations and isolated segments (Dahl et al., 1973; Bleys et al., 1996a). NK1 receptor immunoreactive fibres have been demonstrated in all meningeal compartments, agreeing with our findings (Edvinsson and Uddman, 1982; Edvinsson et al., 1983; Saito et al., 1987; During et al., 1990, 1995; Fricke et al., 1997, 2001). During et al. (1990) who demonstrated the topography and distribution of SP-LI nerve fibres in the rat dura mater encephali suggested that SP occurred only in C-fibres. SP and CGRP-immunoreactive terminals were seen in the leptomeningeal connective tissue (Fricke et al., 1997). SP and CGRP-immunopositive terminals never form neuromuscular junctions (Fricke et al., 1997. There are strong experimental evidences that CGRP- and SP-immunoreactive nerve fibres in the trigeminal system have mechanical, nociceptive, and chemoreceptive functions (Duckles and Buck, 1982; Moskowitz et al., 1982). It has been consistently shown that a selective blockade of NK1 receptors decreases c-fos response within the caudal trigeminal nucleus after mechanical, chemical or electrical stimulation of the trigeminovascular system (Koganemaru et al., 2000). Chemical irritants and autologous blood injected into the subarachnoid space activates trigeminal brain stem neurones, and lead to a significant increase in c-fos expression in the rat trigeminal nucleus caudalis (Nozaki et al., 1992 a, b). Since in vitro studies have revealed that SP and CGRP have a dilatory effect on cerebral vessels, these peptidergic nerve fibres have been thought to be involved in the regulation of the vascular tone (Edvinsson and Uddman, 1982; Edvinsson et al., 1983; Escott et al., 1995). Two arguments contradict the hypothesis that this effect is relevant in physiological conditions. Firstly, SP-immunoreactive and CGRP-immunoreactive nerve fibres are predominantly localized in the outermost layer of the adventitial leptomeninges far away from the smooth muscle cells. Secondly, myoneural synapses built up by the dense plexus of vasodilatory and vasoconstrictive autonomic fibres fulfill these demands much more precisely.

In the present study, a majority of the nitric oxide synthase immunoreactive fibres were associated with the anterior meningeal artery and its branches agreeing with those studies (Estrada, 1993; Berger et al., 1994; During et al., 1995; Saxon and Beitz, 1996). NOS- and NPY-immunoreactive nerve fibres and terminals are restricted to the adventitial leptomeninges of the main cerebral arteries (Estrada, 1993; During et al., 1995). However, the majority of efferent nerve fibres such as NPY and NOS-immunoreactive nerve fibres terminate as neuromuscular synapses on the smooth muscle cells (Nielsen et al., 1971; Fricke et al., 1997). The 5-HT₂B receptor is located on endothelial cells of meningeal blood vessels where it couples to NOS, promoting the local release of NO (Martin et al., 1992; Schmuck et al., 1996). NO is known to excite and sensitize perivascular trigeminal afferents and release of sensory neuropeptides (Yonehara and Yoshimura, 1999). The
study of Szmydynger et al., (1996) has revealed that NO might be involved in the regulation of cerebrospinal fluid production. According to the nicotinamide adenine dinucleotide phosphate (NADPH) reaction, NO containing nerve fibres, as well as NO produced by choroid epithelium itself seem to be involved in those processes.

In the current study, IB4 lectin immunoreactive nerve fibres that run in all directions were distributed in the adventitial meninges of the cerebral arteries. Several differences have been demonstrated in the localization tested glycoconjugates between pial capillaries, small, medium-size and large pial arteries of the control and hypertensive rats (Szumanska and Gadamski, 1992). This difference could be associated with the different functions of particular segments of the pial vascular network. Release of SP and CGRP from peripheral terminals of primary afferents causes vasodilation and plasma extravasation which are considered to be components of the inflammatory response. This correlates well with the close anatomical relationship between CGRP peripheral axons and blood vessels (Silverman and Kruger, 1988). The different GSA IB4 laminar distributions of separate afferent systems probably activate different, although overlapping, pools of second order neurons when noxious sensory stimuli are applied (Ambalavanar and Morris, 1992).

The functional significance of peptidergic nerve fibres becomes evident in the context of neurogenic inflammation, which is regarded as an important experimental model for migraine. SP-immunoreactive and CGRP-immunoreactive nerve fibres are less prominent in the posterior longitudinal ligament of the rat. The terminals of these fibres may act as high threshold mechanoreceptors or nociceptors. The SP-immunoreactive and CGRP-immunoreactive fibres that come in close contact with the vascular wall may be involved in neurogenic inflammation (Lembeck et al., 1982). SP-immunoreactive nerve fibres are obviously less dense than those in the dura mater (During et al., 1990). This observation may be related to the hypothesis that antidromic activation of sensory neurones induces neurogenic vasodilatation and extravasation mediated by SP in the rat leptomeninx. If neurogenic vasodilatation mediated by CGRP, released as a result of Aδ fibre activation, rather than neurogenic extravasation mediated by SP released from C fibres, is a key event in producing or sustaining migraine headache pain, a CGRP receptor antagonist would be an attractive antimigraine target (Williamson and Hargreaves, 2001). Thus, since signalling pain is such a key survival mechanism, it may well be that blocking any signal component of the nociceptive transmitter array will be insufficient to provide relief in severe pain states. The contents of neurokinin 1 receptor-LI and nitric oxide synthase-LI in sensory axons that reach the central nervous system through the ventral root indicate that ventral root afferents may be involved in sensory mechanisms, such as the ventral root pain reaction, as well as in the control of the pial blood vessels. IB4 lectin might provide a powerful tool for studying the neurochemistry and plasticity of nociceptive cutaneous afferents. Chemical activation and sensitisation of meningeal afferents have been shown to be crucial steps in a sequence of events that finally initiate and/or maintain the sensation of headache. Finally, taking into account the variability of neurotransmitter and neuromodulator...
synthesis, and their colocalization and switching under pathological conditions (Hokfelt et al., 1994), further research is necessary to elucidate the relationship of function to localization and evaluate their significance in pathophysiological mechanisms of back pain.

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LOKALIZACIJA RECEPTORA ZA NEUROKININ 1, LEKTIN IB4 I SINTETAZU AZOT OKSIDA U PREPARATIMA MOŽDANIH OVOJNIKA PACOVA NAKON ŠTETNE STUMULACIJE

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SADRŽAJ

U ovoj studiji su vršena ispitivanja imunocitohemijskih karakteristika i distribucije nervnih vlakana i njihovih završetaka u moždanim ovojnicaima 15 Wistar pacova izloženih štetnoj perifernoj stimulaciji. Za ovu ispitivanja su korišćena antitela protiv neurokinin 1 receptora, NO sintetaze i lektina IB4 a zatim je primenjen avadin-biotin-peroksidaza metod. Navedeni neuronski markeri su bili dоказani u svim amijelinskim vlaknima moždanih ovojnica. Nervna vlakna koja su bile pozitivna na lektin IB4 i neurokinin 1 receptor su bile lokalizovane u svim leptomeningevalnim odeljcima gde su se završavala u blizini subarahnoidalnih prostora ili samog vezivnog tkiva. U leptomeningevalnim strukturama i tvrdoj ovojnici mozga zapažena su vlakna koja su bile pozitivna na NO sintetazu i brojna populacija mast čelija. Prisustvo vlakana koja liče na nocepcitne strukture ukazuje na njihovu moguću ulogu u patofizijologiji boli. Proučavanja ove vrste mogu biti od koristi za bolje upoznavanje patoloških procesa na možanim ovojnicaima i njihovoj vaskularnoj mreži.