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Extracranial projections of meningeal afferents and their impact on meningeal nociception and headache

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ABSTRACT

Headaches can be evoked by activation of meningeal nociceptors, but an involvement of pericranial tissues is debated. We aimed to examine a possible extracranial innervation by meningeal afferents in the rat. For in vivo neuronal tracing, dextran amines were applied to the periosteum underlying the temporal muscle. Labeling was observed 2 days later in the parietal dura mater, trigeminal ganglion, and spinal trigeminal nucleus with confocal and electron microscopy. In the hemisected rat head, extracellular recordings were made from meningeal nerve fibers. Release of calcitonin gene-related peptide (CGRP) from the cranial dura mater during noxious stimulation of pericranial muscles was quantified. In vivo capsaicin was injected into the temporal muscle while meningeal blood flow was recorded. In the parietal dura mater, labeled C- and Aδ fibers ramified extensively, accompanied the middle meningeal artery, and passed through the spinosus nerve into the maxillary and mandibular, but not the ophthalmic division of the trigeminal ganglion. Some fibers could be traced into the ipsilateral spinal trigeminal nucleus. Electrophysiological recordings revealed afferent fibers with mechanosensitive receptive fields both in the dura mater and in the parietal periosteum. Noxious stimulation of the temporal muscle caused CGRP release from the dura mater and elevated meningeal blood flow. Collaterals of meningeal nerve fibers project through the skull, forming functional connections between extra- and intracranial tissues. This finding offers a new explanation of how noxious stimulation of pericranial tissues can directly influence meningeal nociception associated with headache generation and why manual therapies of pericranial muscles may be useful in headaches.

1. Introduction

The innervation of the cranial dura mater with trigeminal nerve fibers, first described centuries ago by anatomists like Arnold (1831) and Luschka (1856) [2,39], is regarded as pivotal for the generation of headaches. Neuroanatomical studies demonstrated the close relationship between meningeal blood vessels and nerve fibers of different origin: trigeminal fibers originating in sensory (trigeminal) ganglia [46,48,60], sympathetic fibers from the superior cervical ganglion, and parasympathetic fibers from the sphenopalatine and otic ganglia [36–38,46]. Immunohistochemical studies identified the neuropeptides substance P and calcitonin gene-related peptide (CGRP) in trigeminal afferents of rodents and carnivores, thus discriminating them from autonomic efferents [20,21,32,41,46]. Electrical or chemical stimulation of meningeal afferents provoked the release of CGRP from their peripheral endings [18,19,27]. A considerable proportion of meningeal fibers responded to capsaicin, indicating the presence of vanilloid-sensitive transient receptor potential (TRPV1) channels [17,18]. Electron microscopic examinations on the cranial dura mater proved the existence of myelinated (Aδ) and unmyelinated nerve fibers and classified them according to their vesicular content into afferent and autonomic [1,40,60].

The primary role of the meningeal sensory innervation in generating headaches fits very well to the intraoperative studies of Ray and Wolff and other groups [48,51,65], in which electrical, thermal, or chemical stimulation of meningeal vascular structures caused headache-like sensations. Animal experiments with recordings of action potentials from trigeminal nerves [5] and the trigem-

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inal ganglion [14,59], as well as higher neurons in the medullary dorsal horn [8,35,57] and in the thalamus [11], provided further evidence for an important role of the trigeminovascular system in meningeal nociception and headaches.

Most recently, a role for the pericranial afferent innervation in headache generation is again a matter of discussion [47]. Histological examinations by Kosaras et al. [33] in the mouse have revealed peripherin- and CGRP-immunopositive nerve fibers traversing the bones of the calvaria between the galea aponeurotica and the meninges. These experiments have revived the discussion in the light of the old observations of bone-penetrating dural nerve fibers in humans by Luschka [39] and in monkeys by Penfield and McNaughton [48]. The historical intraoperative data from Ray and Wolff, who observed that noxious stimulation not only of dural but also extracranial structures like pericranial muscles and arteries can cause headache, support this concept [51]. Likewise, further experimental and clinical observations indicated that noxious activation of afferents in pericranial tissues, particularly in the temporal and occipital-cervical regions, can contribute to headache generation [9,31,51,61] and peripheral sensitization in migraine pain [7].

In the present study we employed in vivo neuronal tracings and electron microscopic examinations in rat skulls combined with different functional measurements to investigate extracranial projections from meningeal nerves and their origin in the trigeminal ganglion. A variety of measurements confirmed the afferent nature of extracranial afferent collaterals and their impact on the intracranial secretion of neuropeptides and arterial dilatation. These data clearly show functional afferent connections between intra- and pericranial tissues and provide a new view on the influence of extracranial meningeal afferent projections on meningeal nociception and headache generation.

2. Materials and methods

For all animal experiments, male adult Wistar rats (body weights 200–380 g) were used. Animal housing and all experimental procedures were carried out in compliance with the guidelines for the welfare of experimental animals stipulated by the Federal Republic of Germany. Experimental protocols for in vivo experiments were reviewed by the local district government.

2.1. In vivo tracing

Fourteen male Wistar rats weighing 200–300 g were used. They were initially anesthetized in a closed box by supplying 5% isoflurane (Forene, Abbott, Wiesbaden, Germany). All surgical procedures were performed under general anesthesia using intramuscular injections (each 3 mg/kg body weight) of ketamine (Pfizer, Berlin, Germany) and xylazine (KVP Pharma, Kiel, Germany) and inhalation of 2% isoflurane through a mask. Surgery was performed under aseptic conditions as far as possible. Postoperatively, the animals received metamizole (Ratiopharm, Ulm, Germany) offered in the drinking water (dose calculated to 10 mg/kg body weight in the expected drinking volume).

The temporal muscle was longitudinally split, and an incision of 1 mm length was made into the underlying periosteum cranially of the supramastoid crest and close to the nuchal crest of the occipital bone. Lower-molecular-weight lysine-fixable dextran amines (3000 MW) are taken up by damaged axons and transported preferentially in retrograde direction [25,52]. These tracers label also axon collaterals and nerve fiber terminals [10,52,66] and can even travel transsynaptically [45,49,54]. In 7 rats, a crystal of biotinylated dextran amine (BDA, 3000 MW; Molecular Probes, Eugene, OR, USA) and in the other 7 animals, a crystal of Texas Red-conjugated dextran amine (Texas Red, 3000 MW; Molecular Probes) was placed in the incision of the temporal muscle through a glass capillary [26,50,52]. While the contrast-enhanced BDA technique enables precise labeling of the finest nerve endings and the examination of electron microscopic images, the advantage of the Texas Red fluorescence is that it is easily visible in thick whole mounts like the trigeminal ganglion.

Five minutes after the application, the hygroscopic tracer crystal was dried and the application site was covered with a piece of gelatin sponge (Abgel; Sri Gopal Labs, Mumbai, India) and parafilm to avoid spreading of the dye [62]. Overlying muscle and skin were closed by a suture using a sterile thread. After a survival period of 48–52 h, the rats were deeply anesthetized with an intraperitoneal injection of pentobarbital and transcardially perfused with 250 mL saline (NaCl 0.9%) followed by 200 mL of 2.5% glutaraldehyde if labeled with BDA or 4% paraformaldehyde dissolved in 0.1 M phosphate-buffered saline (pH 7.4) in the case of Texas Red-conjugated dextrans. The dura mater of the middle cranial fossa, the trigeminal ganglia, and the brainstem together with the cervical spinal cord were removed. The trigeminal ganglia were observed as whole mounts using a fluorescence stereomicroscope (Leica MZ FLIII, Leica Microsystems, Bensheim, Germany) with a Texas Red filter set (Ex 560/40 Em 610 LP), and micrographs were taken with a Leica DFC 320 digital camera system. The brainstem with the cervical spinal cord and the trigeminal ganglia were then placed in phosphate-buffered saline containing 30% sucrose at 4 °C for 24 h, quickly deep-frozen in fluid nitrogen, and cut into 20-μm longitudinal sections using a cryostat (CM 3050 S; Leica, St. Gallen, Switzerland). BDA was visualized using the Avidin–Biotin–Peroxidase (Vector Laboratories, Burlingame, CA, USA) method and nickel intensification of the 3,3′-diaminobenzidin (DAB) reaction product [52]. The dura mater and the sections of the trigeminal ganglia as well as of the brainstem with the cervical spinal cord were mounted onto glass slides and coverslipped with fluoromount (Science Services, München, Germany). The labeled sections were examined with a confocal laser scanning system (LSM 710, Carl Zeiss MicroImaging, Jena, Germany) using epifluorescence with a 561-nm Laser and the Rhodamine Red-x filter set for viewing the fluorescent dye and the light transmission mode for viewing the DAB reaction product. The number of labeled neurons in the trigeminal ganglion was counted in the whole mounts and confirmed at higher magnification in the ganglion sections. Micrographs taken from all sections assured that double counting of neurons was avoided. The size of the labeled neurons was assessed by calculating the mean diameter from the longest and the shortest diameter of the cell shape.

The number and the composition of retrograde labeled axons were examined using electron microscopy. In 5 rat heads after the DAB reaction, distal sections of the spinosus nerve identified to be labeled were dissected from the dura mater. The nerve segments were rinsed in phosphate buffer overnight and postfixed in 2% osmium tetroxide, dehydrated through an ascending ethanol series, infiltrated with an ethanol/acetone mixture, pure acetone, acetone/Epon mixture, and finally, embedded in Epon 812. Ultrathin cross-sections (60 nm) were cut with an ultramicrotome (Ultracut E; Reichert Jung, München, Germany), placed on copper meshgrids coated with Pioloform (Plano, Wetzlar, Germany) and examined with a Zeiss 906 electron microscope (LEO, Oberkochen, Germany). The number of BDA-labeled myelinated axons and Remak bundles with unmyelinated axons was counted in all visible peripheral nerve fiber bundles. For the rough assessment of fiber sizes, the smaller diameter of axons was measured.

2.2. Electrophysiological recordings ex vivo

Rats were killed in a CO_2 atmosphere. The head was separated from the body and skinned, the mandible was removed, and the