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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

skull was hemisected in the sagittal plane. The cranial vault was cleared of the pericranial muscles, leaving the periosteum and the insertions of the muscles at the periosteum intact. One skull half was fixed in a perspex chamber such that the skull could be hinged to get simultaneous access to dura and the periosteum. The tissue was perfused continuously at approximately 6 mL/min with physiological solution (synthetic interstitial fluid [SIF], composition in mM: 145 Na^+, 3.5 K^+, 1.53 Ca^{2+}, 0.69 Mg^{2+}, 1.67 $PO4^{2-}$, 114 Cl^-, 9.64 $C_6H_{11}O_7$, 5.55 D(+)-glucose; 7.6 D(+)-sucrose, buffered to pH 7.4 with carbogen gas (95% O_2, 5% CO_2). The temperature of the perfusion solution was controlled with a flow-through Peltier element regulated by feedback from a thermocouple positioned in the bath and in close apposition to the dura. Typically, the preparation was held at 30 ± 1 °C.

The spinosus nerve was identified, cut distally to its point of entry into the trigeminal ganglion, and freed of surrounding tissue over a length of approximately 4 mm. A glass recording electrode (tip diameter ~20–50 μm) filled with physiological solution was attached aside the mobilized nerve by light suction. Signals were filtered (5 kHz low pass), amplified (Axopatch 200A; Axon Instruments, Foster City, CA, USA), digitized (Micro 1401, Cambridge Electronic Design, Cambridge, UK), and stored to disk for later processing. Receptive fields in the periosteum and the cranial meninges were initially localized by von Frey filaments (10 mN, 0.2 mm diameter). For precise mechanical stimulation, a custom-made combined mechanical and electrical stimulator was used. Brief mechanical stimuli of 10 ms at suprathreshold forces (minimum 2-fold) generated single action potentials. The constant latency of electrically or mechanically evoked action potentials allowed the identification of single fibers. Latency delays occur during spontaneous or additionally evoked activity, called "marking" in microneurography [63].

2.3. CGRP release from the cranial dura mater

Rats were killed in a CO_2 atmosphere. The head was separated from the body and skinned, the mandible was removed, and the skull was hemisected in the sagittal plane, but periosteum and pericranial muscles were preserved. The brain was gently lifted out of each of the skull halves so that the adhering cranial dura mater was untouched. The skull halves were superfused at room temperature for 30 min with 500 mL SIF, then placed in a humid chamber above a water bath to maintain a temperature of 35 °C. A 26-gauge cannula connected via a thin tube with a microsyringe was introduced close to the nuchal crest of the occipital bone deep into the temporal muscle or into the insertion of the splenius and longissimus capitis muscles. The injection system was filled with capsaicin (Sigma–Aldrich, Taufkirchen, Germany) dissolved in ethanol and diluted in SIF to a final concentration of 1 μM. The dura lining the cranial cavity was washed 5 times with 350 μL SIF. For the experiment, the skull halves were repetitively filled with SIF collected at intervals of 10 min, from which samples of 200 μL solution were taken for CGRP analysis: 2 baseline samples, the test sample following injection of capsaicin solution (20 μL) into the muscles, and 2 poststimulation samples. To exclude the possibility that capsaicin possibly diffusing through the skull may directly activate nerve fibers in the dura, the TRPV1 receptor antagonist capsazepine (Sigma–Aldrich, Taufkirchen, Germany) dissolved in dimethyl sulfoxide (stock solution of 100 mM) and diluted in SIF to a final concentration of 1 μM was filled into the cranial cavity instead of SIF. In order to ensure that the dural nerve fibers had been responsive to capsaicin, at the end of each experiment the capsazepine was removed from the skull cavity and capsaicin at a concentration of 0.1 μM was added to the SIF for an additional release sample. The skulls that did not show CGRP release after the direct application of capsaicin to the dura were excluded from analysis. In experiments with electrical stimulation, instead of a cannula, a pair of needle electrodes was introduced into the temporal muscle (one electrode placed medially, the other occipitally). Square pulses of 1 ms at 5 mA were applied with a constant current device (WPI A360; World Precision Instruments, Hertfordshire, UK) over 10 min at 10 Hz for intervals of 4 s, interrupted by intervals of 6 s.

The samples were immediately transferred to Eppendorf cups containing enzyme-linked immunosorbent assay (ELISA) buffer with peptidase inhibitors (SPIbio, Paris, France), stored at −20 °C and analyzed on the following day using ELISA kits for immunoreactive CGRP (SPIbio) according to the protocol of the manufacturer. The antibodies are directed against human α/β-CGRP and have full cross-reactivity to rat CGRP with a detection limit of about 2 pg/mL. The optical density was measured using a microplate photometer (Opsys MR; Dynex Technologies, Denkendorf, Germany). Absorbance values were calculated through an interpolation method using a standard curve with defined CGRP concentrations, and blanks without CGRP as control. Data were statistically analyzed using analysis of variance (ANOVA) with repeated measurements extended by Tukey's HSD (honestly significant difference) post hoc test (StatSoft, Inc. 2005, STATISTICA data analysis software system, version 7.1). Differences were considered significant at $P < 0.05$.

2.4. In vivo blood flow measurements

Rats were initially anesthetized in a closed box by supplying 5% isoflurane, continued by 2.5% isoflurane first delivered through a mask and, after quick tracheotomy, through the tracheostoma. Femoral artery and vein were cannulated for recording arterial pressure and supplying substances intravenously (i.v.). When the animal was placed in a frame with the head fixed by ear and mouth bars, it was ventilated with room air enriched by oxygen and 2% isoflurane. A cranial window was drilled into the parietal bone to expose the ramified middle meningeal artery. Optical needle-type probes of a laser Doppler flowmeter (Moore DRT4; Moore Instruments, Axminster, UK) were positioned on branches of the middle meningeal artery with a liquid bridge between the tip of the probe and the dural surface. The meningeal blood flow was continuously recorded [17]. When the blood flow was stable, 20 μL saline or capsaicin solution (dissolved in ethanol as a stock of 10 mM and diluted with saline to a final concentration of 1 μM) were slowly injected within 10 s with a 27-gauge needle deep into the rostral third of the temporal muscle. In one set of experiments, the ganglion blocker hexamethonium chloride (20 mg/kg dissolved in saline) was slowly i.v. injected; in other experiments the CGRP receptor antagonist $CGRP_{8-37}$ (100 μM dissolved in saline) was topically applied to the exposed dura 4 min before injection of capsaicin. $CGRP_{8-37}$ has been shown to reduce the evoked meningeal blood flow within 1–2 min after topical application [34]. Meningeal blood flow (arbitrary units) was measured for 4 min and compared with the mean of a 4-min baseline period before capsaicin injection (mean ± SEM). One-way ANOVA extended by Tukey's HSD post hoc test (Statistica 7.0; StatSoft, Tulsa, OK, USA) was used to compare flow values after capsaicin injection with the baseline. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. In vivo retrograde tracing

We performed in vivo neuronal tracings by applying lower-molecular-weight dextran amines to the pericranial periosteum of the temporal muscle in 14 rats. Retrograde traced nerve fiber

bundles were observed to penetrate the skull through emissary canals and sutures between the temporal and the occipital bone. The penetrating nerve fiber bundles entered the cranial dura of the middle cranial fossa (Fig. 1A), followed branches of the middle meningeal artery, and joined the spinosus nerve in its course towards the trigeminal ganglion (Fig. 1B). Along the whole peripheral course, the retrograde traced nerve fiber split up into smaller bundles and single nerve fibers that showed dichotomous branching and ran within the parietal dura mater far into rostral directions, thereby innervating dural arterial vessels and the dura mater between blood vessels (Fig. 1A–C). The endings of these fine nerve fibers show typical terminal varicosities, as seen in previous examinations [41] (Fig. 1C, D).

In the electron microscopic preparations, both myelinated and unmyelinated labeled axons were found in distal meningeal branches (Fig. 1E) as well as in the proximal spinosus nerve. About 5% (6–10) of the myelinated axons and about 10% (25–35) of unmyelinated axons and Remak bundles in all identified peripheral nerve fiber bundles were retrograde labeled with biotinylated dextran amines (n = 5 animals). The diameters of the myelinated axons (including myelin sheath) ranged from 1.2 to 5.2 μm. Since Remak bundles are composed of several unmyelinated axons, which are rarely visible separately after DAB labeling (Fig. 1E), the total number of labeled axons is certainly much higher than the number of the counted profiles.

Before the spinosus nerve enters the mandibular division of the ganglion, it usually splits up into 2–3 branches (Fig. 2A). In 10 rats (7 labeled with Texas Red, 3 with BDA), the cell bodies of labeled nerve fibers were observed in the trigeminal ganglion. In all cases, neurons were found preferably in the mandibular division, some in the maxillary division, but none in the ophthalmic division of the trigeminal ganglion (Fig. 2A). The number of labeled bodies ranged from 30 to 45 and had a size of 15–40 μm (mean diameter) (Fig. 2B–D). In the distal mandibular and maxillary nerves, no

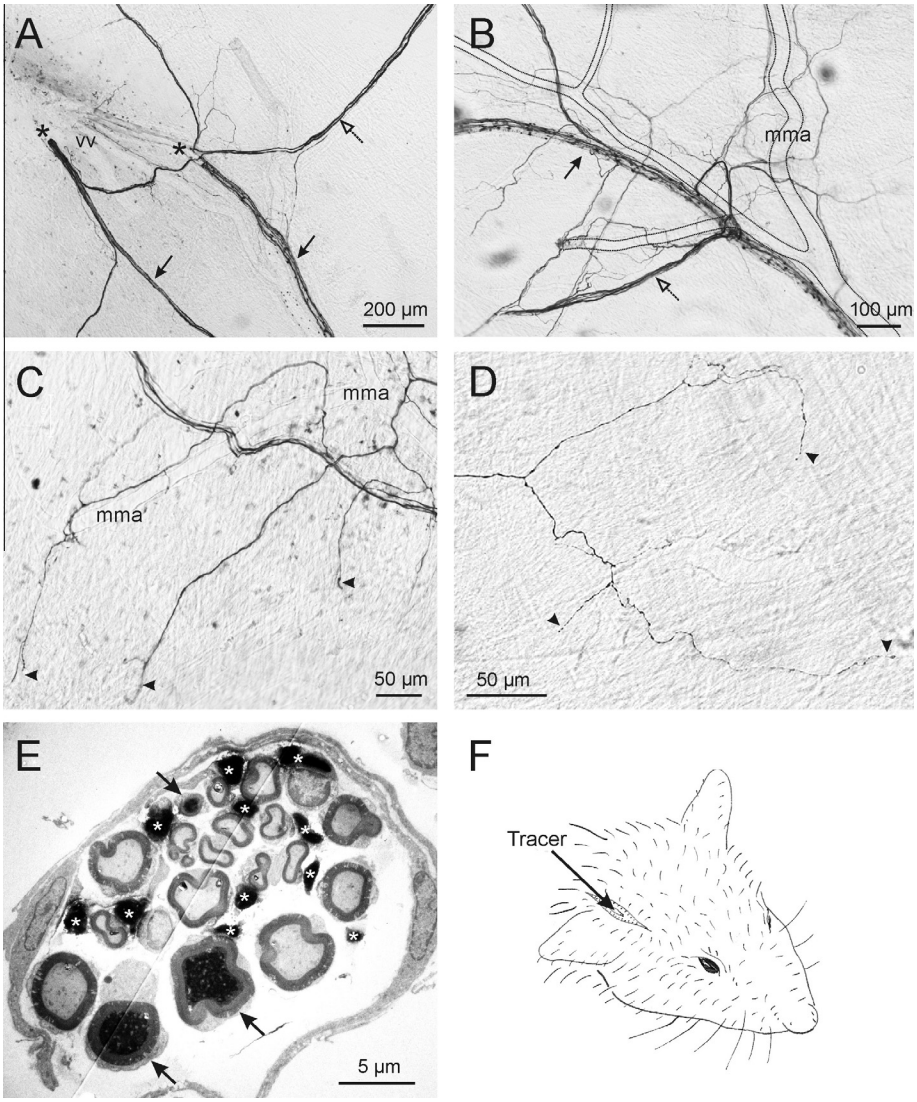

Fig. 1. Nerve fibers labeled by lower weight dextran amines (Tracer), applied to the periosteum underlying the temporal muscle (F), visualized by the nickel intensified black DAB (3,3′-diaminobenzidin) product in the isolated dura mater. (A) Two bundles of retrograde labeled nerve fibers (arrows) are disconnected at the site of their entrance (*) along emissary venous vessels (vv) through the skull into the dura mater. Collateral nerve fibers branching off from the retrograde labeled nerve fiber bundles run in rostral direction (blank arrow). (B) Bundle of retrograde labeled nerve fibers (arrow) approaching the middle meningeal artery (mma; visible by its meandering course) gives off (in anterograde direction labeled) nerve fiber bundles (blank arrow) and multiple collateral fibers. The shape of the mma is pointed out by a dotted line. (C) Labeled nerve fiber bundles and single fibers accompanying and crossing branches of the mma; some of them form terminals in dural connective tissue (arrowheads). (D) Fine-labeled collateral fiber forming several terminals (arrowheads) in the dural connective tissue. (E) Electron micrograph of a cross-section through a labeled peripheral nerve fiber bundle containing 3 myelinated axons filled with the tracer (arrows) and 11 stained Remak bundles (stars), each presumably containing several unmyelinated fibers.

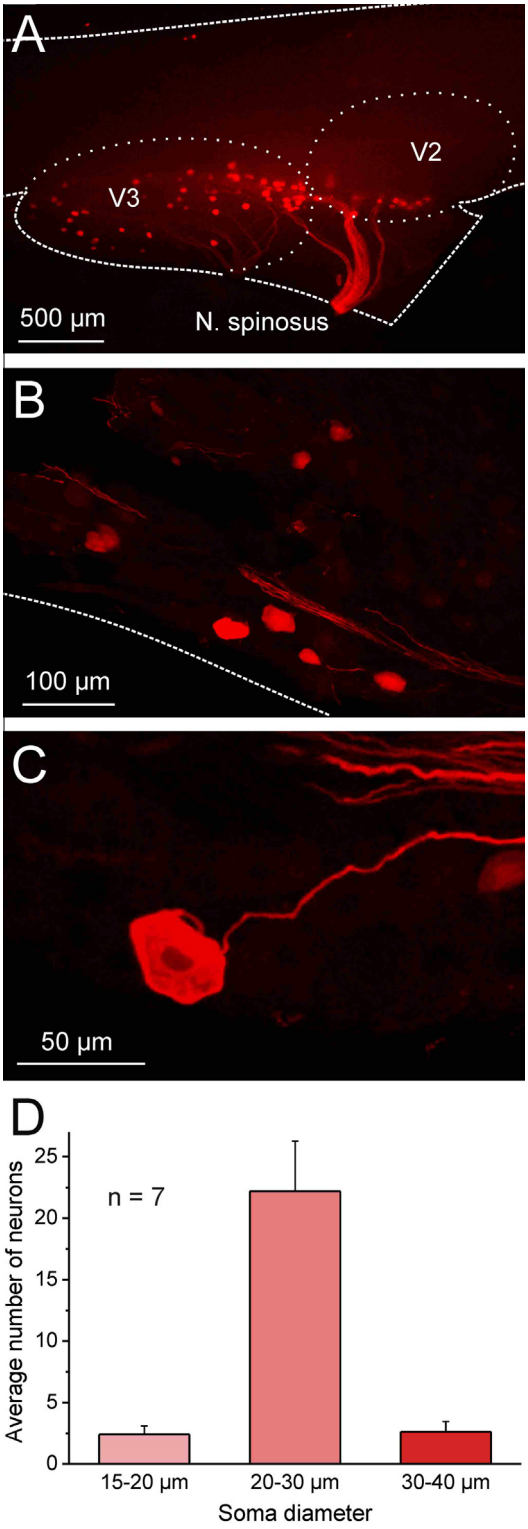

Fig. 2. Retrograde Texas Red labeled nerve fibers and neuronal somata in trigeminal ganglion. (A) Whole mount micrograph of a trigeminal ganglion (dashed contour). The labeled spinosus nerve (*N. spinosus*) divides into 2 or 3 parts before entering the trigeminal ganglion. Labeled neurons are preferably found in the mandibular division (V3) and to a moderate extent in the maxillary (V2) division of the trigeminal ganglion but not in the ophthalmic division. (B) Confocal micrograph of labeled neuronal cell bodies of different size, partly connected with nerve fiber processes. (C) Confocal micrograph of a large labeled neuron with nerve fiber process. (D) Distribution of soma sizes (mean ± SD) of labeled neurons in 7 rats.

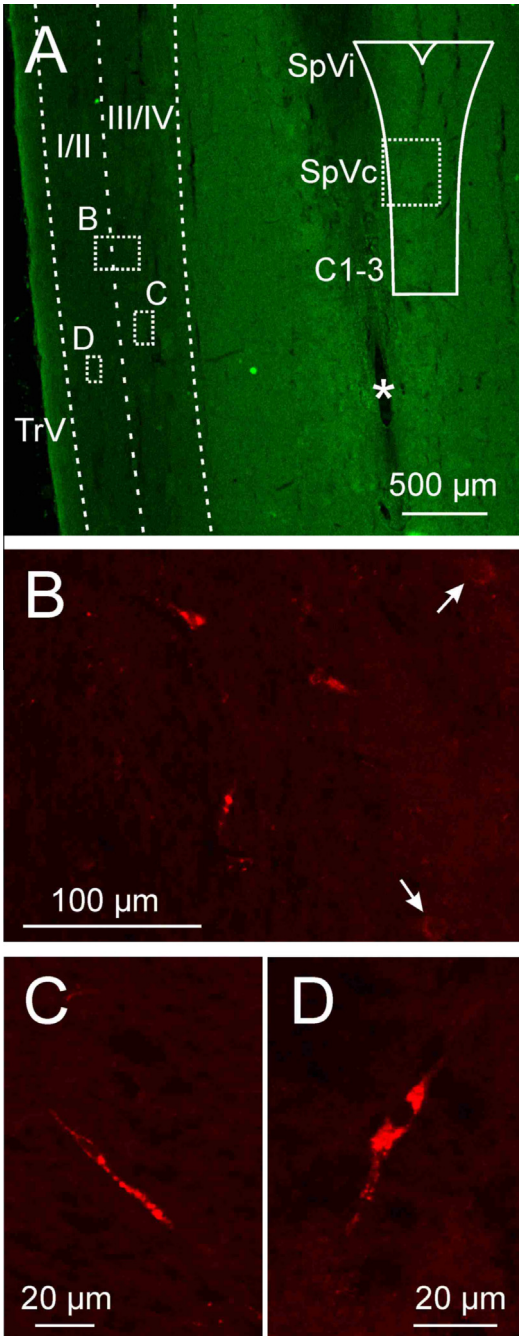

Fig. 3. Transganglionic labeling of neuronal structures in the ipsilateral spinal trigeminal nucleus caudalis (SpVc). (A) Horizontal section through the SpVc visualized in the green channel for contrast enhancement (Laser 488 nm, fluorescein isothiocyanate [FITC] filter set); the central canal is obliquely cut (∗). The schematic overlay shows the contours of the brainstem with the location of the subnucleus interpolaris (SpVi), caudalis (SpVc), and the C1-3 cervical segments. The contours within the background image (broken lines) define the spinal trigeminal tract (TrV) and the superficial (I/II) and deep laminae (III/IV) of the SpVc with the position of the labeled neurons in B–D. (B) Labeled spindle-like neurons at the border of Lamina II and III. Note not labeled larger round neurons in the background (arrows). (C) Labeled neuron with long processes in lamina III. (D) Labeled neuron in lamina II of the SpVc.

stained nerve fibers were detected, indicating the lack of contamination by diffusion of the dye along other structures than branches of the spinosus nerve.

In 7 rats treated with Texas Red-conjugated dextran amines, transganglionic labeling of structures in the ipsilateral trigeminal

nerve was detected. Labeled fibers were found in the ipsilateral spinal trigeminal tract, and some (transsynaptically) labeled spindle-shaped neurons were detected in the spinal trigeminal nucleus (Sp5c), specifically in the caudal part (Fig. 3A–D). Labeling was seen both in the superficial (laminae I and II) and in deeper layers (laminae III and IV) of the Sp5C (Fig. 3). No labeled fibers and neurons were detected on the contralateral side or in the cervical (C1 to C3) spinal dorsal horn.

3.2. Electrophysiological recordings from meningeal afferent fibers

Knowing the extracranial innervation sites of the spinosus nerve, we used an in vitro preparation of the hemisected rat skull, formerly described in detail [12], to record extracellularly from afferent axons of this nerve innervating the parietal periosteum beneath the temporal muscle (Fig. 4A). Extracranial receptive fields were located by mechanical probing or electrical stimulation using a combined electromechanostimulator [13]. In 25 recordings from the spinosus nerve, we found 23 receptive fields of single afferents responsive to mechanical stimulation of the periost. According to their conduction velocities, the afferents belonged to C or Aδ fibers, and their mechanical thresholds tended to decrease with increasing conduction velocities (Fig. 4D). In addition to their sensitivity to stimulation of the periosteum, 5 of these afferents could be activated by probing the dura mater, and 6 of 17 tested were activated by capsaicin (1 μM) applied into the bath solution (Fig. 4D). In order to verify that the receptive fields in the dura and in the periosteum were innervated by one and the same axon, we first performed collision experiments stimulating electrically nerve fibers in the periosteum and the dura. In 2 cases, in which only one action potential could be elicited at a certain stimulus interval, the singularity of an axon running through the dura and the periosteum could be verified (Fig. 4B). However, the disadvantage of this technique is that it is not possible to distinguish between sensory and autonomic fibers and to assure the existence of receptive fields in the stimulated tissue. Therefore, we adopted a technique from microneurography, which allows identifying single axons and their activation by tracking the latency of an action potential elicited by periodical stimulation [64]. For the periosteum, instead of electrical stimuli, we used mechanical stimuli with very short duration, which can elicit single action potentials [13]. Figure 3C shows 2 units (Unit 1 and Unit 2) responding after stimulation of the periosteum with a single action potential at constant latency to every single sinusoid mechanical stimulus of 10-ms duration. Between stimulus 3 and 4, a short cluster of discharges was provoked by probing the dural receptive field of Unit 2 (Fig. 4C, horizontal arrow in the vertical recording trace on the right). In stimulus 4, this unit showed a rightward shift in latency (inclined arrow) with slow recovery, while the latency of Unit 1 remained constant (Fig. 4C, left). The increases in latency of the action potentials elicited from the periosteum after stimulation of the dural receptive field is evidentiary for the stimulation of the same axon and let us conclude that collaterals of the same nerve fiber can innervate dura and periosteum. Among the 23 examined afferents, we found such evidence for extracranial collaterals in 5 cases (Fig. 4D).

3.3. Neuropeptide release

Having confirmed the presence of communicating axons with intra- and extracranial mechanosensitive receptive fields, we asked whether stimulated communicating chemonociceptive afferents can release neuropeptides. On this account we examined whether the extracranial activation of capsaicin-sensitive fibers via action potentials invading axon collaterals leads to a significant release of CGRP. This mode of ortho- and antidromic activation known as axon reflex has been suggested to be implicated in migraine pathophysiology [33,42]. We used our established hemisected rat skull preparation, in which CGRP released from the dura mater into the physiological solution filling the cranial cavity can be collected at regular intervals (10 min) and quantified with an ELISA. Stimulation of the intact temporal muscle with electric pulses (1 ms, 5 mA, 10 Hz; $n = 17$) was followed by a significant increase in CGRP concentration in the cranial cavity from the baseline of 10.6 ± 1.8 pg/mL to 33.8 ± 5.6 pg/mL [$F(4,64) = 16.4$, $P < 0.001$; Fig. 5A, Elec stim]. Injection of 20 μL capsaicin solution (1 μM) into the temporal muscle caused an increase from 20.5 ± 3.0 pg/mL to 45.6 ± 8.6 pg/mL [$F(4,40) = 11.4$, $P < 0.001$; Fig. 5A, Caps inject], and injection into the splenius and longissimus capitis muscle caused an increase from 16.0 ± 1.7 pg/mL to 31.5 ± 4.8 pg/mL [$F(4,40) = 12.2$, $P < 0.001$; Fig. 5B]. In the experiments with chemical stimulation of the temporal muscle, the capsaicin (TRPV1) receptor antagonist capsazepine (10 μM) was added to the SIF in the cranial cavity to block a potential direct action of capsaicin on meningeal afferent terminals. The higher baseline in CGRP release compared to the experiments with electrical stimulation is probably due to a partial agonistic effect of capsazepine on TRPV1 receptors.

3.4. Meningeal blood flow

Previous studies have shown that CGRP released from activated meningeal afferents increases meningeal blood flow [34]. In order to test if noxious stimulation of the temporal muscle changes the meningeal blood flow, laser Doppler probes were attached to the middle meningeal artery exposed in the cranial window of anesthetized rats (Fig. 5C). When saline was slowly injected into the ipsilateral temporal muscle, no significant increase in meningeal blood flow was registered, while injection of capsaicin solution (1 μM) into the temporal muscle was followed by a significant increase in blood flow. The mean increase in flow of 7.6 ± 1.5% was significantly different from the basal flow prior to stimulation [1-way ANOVA, $F(3,43) = 15.3$, $P < 0.001$; Fig. 5C]. To exclude the possibility that this increase was mediated by the parasympathetic system, which may be involved during extracranial noxious stimulation [29], rats were pretreated with the ganglion blocker hexamethonium. After i.v. infusion of 20 mg/kg hexamethonium chloride, the capsaicin injection was followed by nearly the same increase in flow (7.2 ± 1.1%), which was again significantly different from the basal flow ($P < 0.001$), confirming that the blood flow changes induced by noxious extracranial stimulation was not controlled by the autonomic nervous system (Fig. 5C). The arterial pressure after hexamethonium infusion fell by 16.3% (±10.2%) on average. When the CGRP receptor antagonist $CGRP_{8-37}$ was topically preadministered to the dura, capsaicin injection into the temporal muscle did not increase the meningeal blood flow. Taken together, activation of chemonociceptors in the temporal muscle and the underlying periosteum with capsaicin caused an increase in meningeal blood flow through the release of the vasodilatory neuropeptide CGRP in the cranial dura mater.

4. Discussion

The pathogenesis of primary headaches like tension-type headache or migraine is still obscure and a matter of ongoing discussion. Arguments for a central origin collide with the view of a peripheral activation of intracranial nociceptive afferents as a primary event in headache generation. The results of our morphological and functional studies demonstrate that collaterals of trigeminal afferents form functional connections between intra-

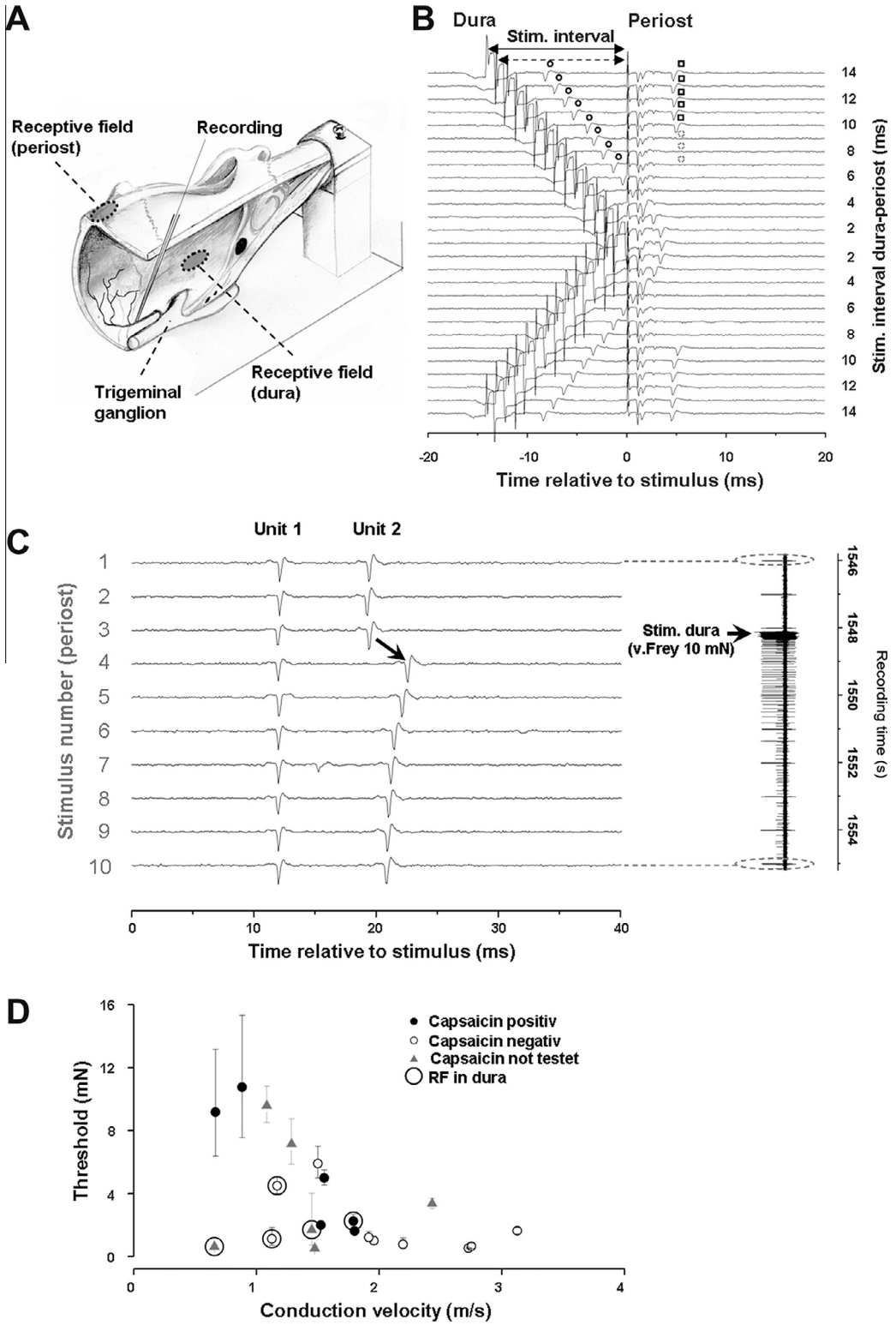

Fig. 4. Electrophysiological recordings in the hemisected rat cranial preparation. (A) Set-up showing recording electrode attached to the meningeal spinosus nerve near the trigeminal ganglion. An extracranial receptive field in the parietal periosteum and an intracranial receptive field in the dura mater are outlined. (B) Electrically evoked action potentials from the dural (circles) and the periosteal receptive field (squares) can be shown to arise from the same axon by exploiting the phenomenon of collision. Reducing the time interval from 14 to 1 ms between the dural and periosteal stimulation revealed that an interval <9 ms resulted in the generation of only one action potential arriving from the stimulated dural receptive field (circle). The action potentials evoked from the periosteal receptive field were extinguished by collision and no response is observed at the expected time point (dotted squares). (C) Repetitive short mechanical stimuli at intervals of 1 s applied to the periosteal receptive field generated 2 action potentials (Unit 1 and Unit 2) with characteristic latencies (about 12 and 20 ms poststimulus). Stimulation of the dural receptive field with a von Frey filament (10 mN) between the periosteal stimulus 3 and 4 caused a burst of action potentials (right panel, horizontal arrow) and a selective increase in latency of Unit 2 (left panel, declined arrow) indicative for an additional activation of exactly this unit (''marking''). (D) Sample of 23 units with their conduction velocities and mechanical thresholds to mechanical stimulation of their extracranial (periosteal) receptive fields. Error bars represent forces between 10% and 90% response probability (for detailed methodological description, see [12]). Six of 15 units tested responded to capsaicin (Capsaicin positive) and 9 were unresponsive (Capsaicin negative). Five units had an additional mechanosensitive receptive field in the dura evidenced by ''marking'' as shown in C.

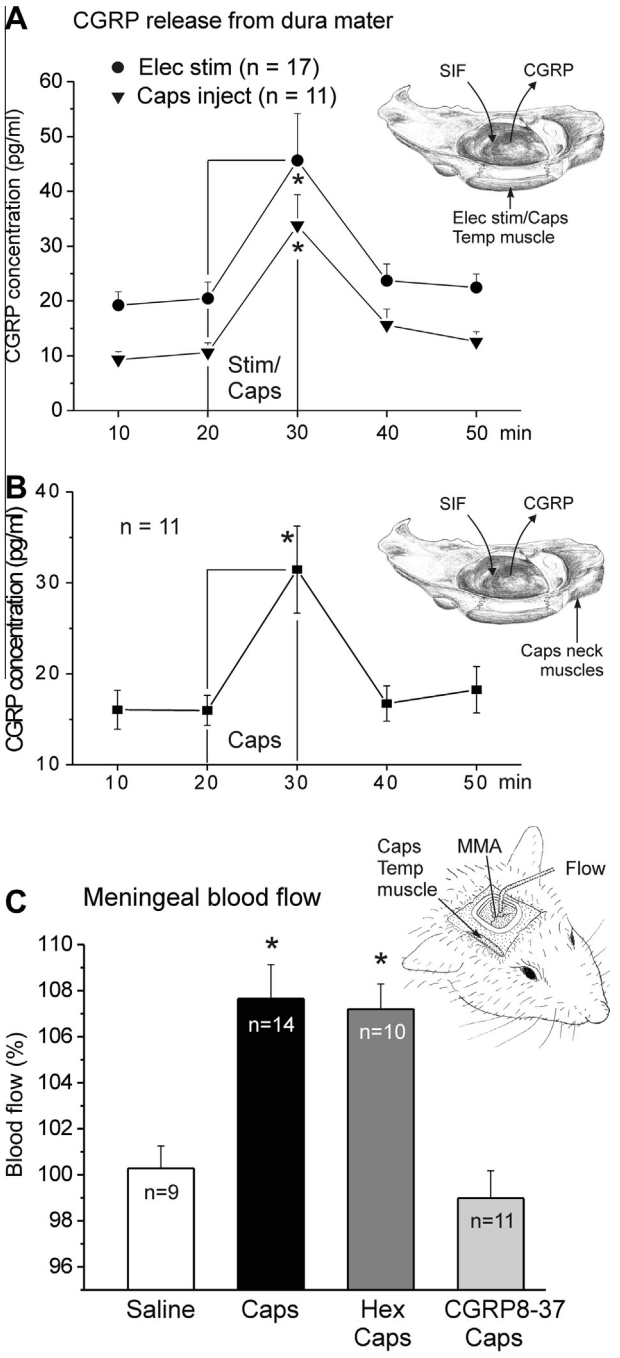

Fig. 5. Neuropeptide release from the dura mater in the hemisected rat cranial preparation (A, B) and meningeal blood flow recordings (C) confirming afferent collateral innervation of intra- and extracranial structures. Calcitonin gene-related peptide (CGRP) concentrations in the synthetic interstitial fluid (SIF) filled into the cranial cavity at 10-min periods are increased after stimulation of the temporal muscle (A) with electrical pulses (Elec stim) or injection of capsaicin (Caps inject) into the temporal muscle (A) or the neck muscles (B). (C) Meningeal blood flow is increased after injection of capsaicin (Caps) into the ipsilateral temporal muscle. The increase is not influenced by intravenous preadministration of hexamethonium (Hex) but blocked by local preapplication of the CGRP antagonist $CGRP_{8-37}$, indicating that it was caused by the vasodilatory action of CGRP released from meningeal collaterals of afferent fibers innervating the temporal muscle.

and extracranial tissues and provide new evidence for an extracranial origin of meningeal nociception. By this way, signals arising in pericranial muscles can reach the dura mater by ortho- and antidromic conduction through axon collaterals, possibly influencing meningeal functions and the generation and management of headaches in humans.

Nerve fibers of the dura mater penetrating the human cranium were first mentioned in the middle of the 19th century by the German anatomist Hubert Luschka [39]. During the 20th century, these observations could be confirmed in humans and in animal models by more advanced histological techniques [15,48]. The innervated structures and the functional significance of these penetrating nerve fibers remained unclear for a long time; researchers followed Luschka's original speculation that these fibers innervate the mastoid. Recently, skull-penetrating nerve fibers connecting the meninges and the periosteum along emissary canals and sutures have been described on the basis of neuronal staining techniques in the mouse [33]. In the present tracing experiments, the passage of nerve fibers through the skull could not be directly demonstrated because decalcification of the bone would have destroyed the dextran amine staining. However, the entrance of retrograde labeled nerve fibers into the dura mater concurred with the localization of the suture between the occipital and temporal bone and with the emissary canals. Together with recent (unpublished) anterograde tracing experiments in rat and human skulls, this suggests a similar innervation principle in all species. The cross-sections of distal nerve bundles visualized on the electron microscopic level revealed that the labeled nerve fiber profiles belong to Aδ and C-fiber classes (Fig. 1E). The estimated number of labeled peripheral axons far exceeds the number of stained neuronal cell bodies in the trigeminal ganglion, supporting the assumption that one neuron can give rise to multiple collateral branches of its peripheral axon.

The results of our morphological and functional studies clearly show that afferent fibers of the trigeminal nerve innervating the cranial dura mater can form functional collateral axons that leave the skull to innervate extracranial structures, as previously suggested [33]. These collaterals can deliver sensory information from the outside of the head to the cranial dura, most likely contributing to meningeal nociception and neuropeptide release in the vicinity of arterial blood vessels causing vasodilatation and increased meningeal blood flow. The increase in blood flow by about 8% is in the range of the blood flow increase of maximally 15% caused by topical application of CGRP at a high concentration (10^{-4} M) [34]. More importantly, however, this response shows that at least some of the collaterals contain and release CGRP, because the blood flow increase could be abolished by blocking CGRP receptors. When the collateral axons are activated from extracranial sites, the activity is conducted into the cranium and antidromically distributed along the intracranial branches. The neuropeptide release occurs probably along the whole terminal branches, which partly accompany the meningeal arterial vessels, as described earlier [41]. Functionally active collateral branches innervating the dura mater are evident not only from the tracing studies, but particularly from the electrophysiological recordings, which reveal receptive fields of identical afferent fibers both in the periosteum and in the dura mater (Fig. 4).

The extracranial tracing, which stained meningeal axons all along the middle meningeal artery through the spinosus nerve into the trigeminal ganglion including somata of ganglion neurons, shows clearly the trigeminal origin of these fibers. Lower-molecular-weight lysine-fixable dextran amines as used in the present study are transported with a velocity of more than 20 mm/day [25]. They allow the sensitive and detailed visualization of axon collaterals and can be easily combined with electron microscopic studies [44,52]. The tracing stained also nerve fibers in the ipsilateral spinal trigeminal tract and in superficial as well as in deep laminae of the spinal trigeminal nucleus caudalis (SpVc), suggesting that this afferent system belongs to the nociceptive trigeminovascular system. The SpVc is primarily involved in transmission and processing of nociceptive information from the facial and cranial areas [16,28,43] and has been identified as the central target of

previous retrograde tract tracings from the middle meningeal artery and the superior sagittal sinus [37,38]. In the trigeminal nucleus, even some stained small second-order neurons could be identified, suggesting that the tracer can partially be transported transsynaptically (Fig. 3).

The biological value of an extracranial innervation by meningeal afferents may be seen in the context of a protective function for the brain. Meningeal nerves consist of primary afferent and autonomic fibers releasing a variety of vasoactive neuropeptides [32], which contribute to the control of arterial vessel tone and hence, perfusion of the dura mater. The control of dural perfusion may play an important role for thermoregulation, which has been the subject of recent critical discussions [6,30]. The blood supply may also subserve inflammatory and immune responses in the dura mater [53]. A major part of meningeal afferents may have nociceptive functions responding to mechanical, chemical, and thermal stimuli [5,58], which can be regarded as a warning system to avoid damage of the brain. We hypothesize that collateral fibers leaving the dura mater to extend their receptive fields to the extracranial compartment can form a fast-acting and efficient system to signal neuronal information about external detrimental influences such as high temperature or tissue damage through the skull to the meninges, leading to protective reactions like increased blood flow and enrichment of immune cells.

It is not yet clear if a similar direct functional afferent connection between extra- and intracranial structures exists also in humans, but the classical intraoperative experiments argues for this possibility [23,51,65]. The clinical relevance of extracranial collaterals of meningeal afferents should be seen in the context of headache generation and may explain clinical findings such as the high prevalence of neck pain in migraine [9] as well as therapeutic approaches in headaches. For example, a case of chronic migraine with refractory pain was reported to be successfully treated with auriculotemporal nerve stimulation [56]. The authors discussed a possible extracranial origin of these headaches, referring to the idea of extracranial collaterals spreading from meningeal afferents [33]. Provided that similar innervation patterns exist in humans, the concept of extracranial collaterals spreading from meningeal afferent fibers has the potency to modify our understanding of headaches. It must be assumed that the intracranial afferent innervation is no longer the only origin for headache generation; moreover, headaches may arise from deep pericranial structures like temporal periosteum, or may be triggered by stimulation of these structures, because the brain is not able to know from where the nociceptive inputs come. Conversely, intracranial pain may also be perceived as extracranial, rendering the whole head painful without clear localization of the pain. This provides not only a potential source for diagnostic error, but may also explain why tension-type headache, for example, is difficult to diagnose clearly and to manage adequately.

So far, based on former studies, neurons of the ophthalmic and maxillary divisions of the trigeminal system were most frequently observed to be involved in migraine and tension-type headache, while we have found nearly all retrograde traced neurons in the mandibular division of the trigeminal ganglion. This surprising finding can be explained by the fact that the parietal area of the dura mater is nearly exclusively innervated by the spinosus nerve, which arises from the mandibular part, while the frontal and apical parts are predominantly innervated by nerve fibers originating in the ophthalmic division following the superior sagittal sinus up to the apical dura. Having already some (unpublished) morphological evidence, we assume that the principle of extracranial collaterals can similarly be found in ophthalmic territories, but the experimental proof is technically more difficult. Nevertheless, the mandibular area may also be important for the pathophysiology of headaches. There is a high incidence of temporomandibular disorders and primary headaches [22], which could directly be influenced by meningeal afferents innervating an affected temporal muscle. Similarly, the association of tension-type headaches and migraine with hardening of neck muscles is well known and may also be influenced by such collaterals [24]. From a therapeutic aspect, physical treatment of neck muscle tension [4] may be useful, as well as blockade of peripheral nerves and so-called trigger points, for example, by local anesthetics [3] or injection of botulinum toxin into pericranial structures [55] for treatment of migraine and tension-type headache. Though the mechanism of these therapies is not clear in any case, these interventions may be speculated to be beneficial because they can directly reduce the nociceptive inflow to the spinal trigeminal system through headache-inducing meningeal afferents with collateral extensions into pericranial tissues.

Conflict of interest statement

The authors declare no conflicts of interest.

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