Research Submission

Innervation of Rat and Human Dura Mater and Pericranial Tissues in the Parieto-Temporal Region by Meningeal Afferents

Markus Schueler, MD; Winfried L. Neuhuber, MD; Roberto De Col, MD; Karl Messlinger, MD

Objective.—To reinvestigate the innervation pattern of the dura mater of rat and human middle cranial fossa, the morpho-functional substrate of headache generation, and adjacent extracranial tissues with neuronal *in vitro* tracing.

Background.—This study was initiated by recent structural and functional findings of meningeal afferent fibers which innervate the cranial dura mater and may project to extracranial tissues.

Methods.—Anterograde and retrograde neuronal *in vitro* tracing was made in formaldehyde fixed hemisected rat and human skulls. The fluorescent tracer DiI was applied to proximally cut meningeal nerves in rat and to distal branches of the spinosus nerve in human calvaria lined by dura mater. After several weeks, the dura mater and deep extracranial tissues were examined with fluorescence microscopy.

Results.—In addition to a network of meningeal nerve fibers, several fiber bundles were observed, leaving the skull through emissary canals and fissures to innervate the pericranial temporal, parietal, and occipital periosteum. Traced fibers were seen spreading into deep layers of the temporal and upper neck muscles. Retrograde neuronal tracing revealed labeled cell bodies exclusively in the mandibular and maxillary division of the rat trigeminal ganglion, and centrally projecting fibers were identified in the spinal trigeminal tract. Electron microscopy of the cross-sected spinosus nerve showed myelinated and unmyelinated axons with similar numbers in human and rat.

Conclusions.—We conclude that a proportion of meningeal afferents innervates extracranial tissues like periosteum and pericranial muscles via collaterals projecting through the skull. These afferents may be nociceptive, some may subserve proprioceptive functions. The finding of extracranial projections of meningeal afferents may be important for our understanding of extracranial impacts on headache generation and therapy.

Key words: neuronal in vitro tracing, DII, fluorescence microscopy, trigeminal ganglion, spinosus nerve, headache

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From the Institute of Physiology and Pathophysiology, Friedrich-Alexander University Erlangen-Nürnberg, Erlangen, Germany (M. Schueler and K. Messlinger); Institute of Anatomy, Friedrich-Alexander University Erlangen-Nürnberg, Erlangen, Germany (W.L. Neuhuber); Department of Anaesthesiology and Operative Intensive Care, Faculty of Clinical Medicine Mannheim, University of Heidelberg, Mannheim, Germany (R. De Col); Department of Nephrology and Hypertensiology, Friedrich-Alexander University Erlangen-Nürnberg, Erlangen, Germany (M. Schueler).

Address all correspondence to K. Messlinger, Institute of Physiology and Pathophysiology, University of Erlangen-Nürnberg, Universitätsstr. 17, D-91054 Erlangen, Germany, email: karl.messlinger@fau.de

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As early as in the middle of the 19th century, the German anatomists von Luschka and Arnold published the first anatomical studies about nerve fibers of the human cranial dura mater.^{1,2} Two generations later, Dowgjallo and Grzybowski localized the origin of meningeal nerve fibers in the trigeminal ganglion Gasseri,^{3,4} followed by the comprehensive work of Penfield and McNaughton on the innervation of the primate and human cranial dura mater.⁵ Using anatomical and histological techniques, they showed that the dura of the middle cranial fossa is innervated by nerve fibers of the mandibular and maxillary

Conflict of Interest: None.

Headache

trigeminal divisions, while the dura of the anterior cranial fossa and the tentorium cerebelli are innervated by nerve fibers of the ophthalmic division.

Due to the seminal intraoperative experiments of Ray and Wolff during this time, the essential role for the generation of headaches of trigeminal nerve fibers innervating meningeal blood vessels was published and widely accepted,⁶ later confirmed by additional studies of other groups.^{7,8} A number of animal experiments during the last decades widened our knowledge about the trigeminovascular system of the meninges as morpho-functional basis for the pathogenesis of headaches. Further studies clarified details of the structure⁹⁻¹² and the functional characteristics of meningeal afferents innervating the rodent dura mater,¹³⁻¹⁶ and identified their central projections.^{17,18} Through immunohistochemical examinations, we know that a considerable proportion of these meningeal afferents contains neuropeptides like substance P and calcitonin gene-related peptide.^{10,19,20} This could also be demonstrated for the human dura mater, confirming the homology of the nociceptive innervation between mammals.²¹

Despite this knowledge, the pathogenesis of headaches is still full of unresolved problems, which applies particularly to primary headaches such as migraine. While a central origin of primary headaches has been intensely discussed during recent years, clinical and experimental observations provide evidence for an essential contribution of peripheral, intracranial as well as extracranial, nociceptive processes.²² The intracranial and pericranial trigeminal innervation may partly form a functional unit, a concept that is supported by recent histological and functional data that show collateral afferent connections between the dura mater and deep pericranial tissues in rodents.^{23,24} In the light of these recent findings, old anatomical studies on the primate and human meningeal innervation reporting about nerve fibers that penetrate the skull become again highly important.^{2,3,5} These nerve fibers, in addition to their originally supposed function to innervate the skull, eg, in the region of the mastoid,⁵ can indeed supply extracranial tissues.

The present postmortem anterograde tracing study in rat and human skulls completes and extends our recent *in vivo* anterograde tracings.²⁴ They show that in the area of the middle cranial fossa, intracranial (meningeal) and extracranial (deep muscular) tissues are innervated by trigeminal nerve fibers passing through the cranial dura mater. In addition, we provide evidence for considerable similarities between rodent and human meningeal innervation regarding the course and the composition of nerves that supply the middle cranial fossa.

MATERIALS AND METHODS

Human skulls were obtained from adult male bodies (age 67, 69, and 90 years) donated to the Institute of Anatomy of the University of Erlangen-Nürnberg. The use of human tissue was approved by the university's ethics committee. For the animal experiments, 25 male adult Wistar rats (body weights 200-380 g) were used. Animal housing and experimental procedures were carried out in compliance with the guidelines for the welfare of experimental animals stipulated by the Federal Republic of Germany.

Preparation of Rat Skulls for Postmortem Neuronal Tracing.—Rats were killed by inhaling CO₂. The head was separated from the body and skinned, the mandible was removed, and the skull was hemisected in the sagittal plane. The brain was lifted out of each of the resulting skull halves, while the adhering cranial dura mater, the trigeminal ganglion, and the brainstem, as well as periost and pericranial muscles, were preserved. The dura mater overlying the meningeal branch of the mandibular nerve (referred to as spinosus nerve) was excised about 2 mm from the trigeminal ganglion (Fig. 1A,B). The spinosus nerve was cut and a small crystal of DiI (1,1'Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate, D282, Molecular Probes, Eugene, OR, USA) was attached both at the distal and the proximal nerve stump for postmortem retrograde and anterograde tracing.²⁵⁻²⁸ Then the application site was covered with a small piece of gelatin sponge (Abgel, Sri Gopal Labs, Mumbai, India) to avoid spreading of the dye.

Preparation of Human Skulls for Postmortem Neuronal Tracing.—Three unfixed human skulls were transected along the sagittal suture and the brain was removed. The adhering dura mater and the extracranial tissues (periost and pericranial muscles)



Fig 1.—Anterograde DiI tracing of rat spinosus nerve. (A) Fenestrated rat skull showing the position of the micrographs (C-G). Through the cranial window, the middle cranial fossa and the trigeminal ganglion (TG) of the contralateral side are visible. The tracer (DiI) was applied to the proximal spinosus nerve close to the TG. (B) Basolateral aspect of the middle cranial fossa showing the course of the spinosus nerve (white broken line), which was dissected and cut (black broken line) close to the TG for the tracer (DiI) application. The spinosus nerve runs first in caudal direction and then accompanies the middle meningeal artery (MMA) in rostro-lateral direction. (C) Basolateral dura mater with the proximal part of the ramified spinosus nerve accompanying the main branches of the MMA (faintly stained in the background). (D) Network of labeled nerve fiber bundles accompanying the MMA in the parietal dura. Some single fibers exhibit terminals (arrowheads). (E and F) Labeled nerve fiber bundles penetrating the cranium; (E) in the fissure between temporal bone (TB) and the parietal bone (PB); (F) in the petrosquamous fissure between the TB and the occipital bone (OB) around the internal maxillary vene (IMV). The green background has been generated by overlying the same image taken with the green (FITC) filter. (G) Network of labeled nerve fiber sin the temporal muscle, (H) nerve fiber bundles and single fibers running along an arterial vessel in the periosteum underlying the temporal muscle. (H and I) Labeled nerve fibers in the temporal muscle, (H) nerve fiber bundle traveling through the endomysium between cross-cut muscle fibers; (I) nerve fiber endings extending between (faintly stained) muscle fibers.

were kept intact. The meningeal branch of the mandibular nerve (spinosus nerve) was located at the entrance of the middle meningeal artery (MMA) into the skull base. The course of this nerve and its branches along the arborized MMA was followed by careful dissection under a stereomicroscope (Fig. 2A,B). Peripheral branches of the MMA together with adjacent nerve bundles were cut, and 3-5 crystals of DiI (1,1'Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate, D282, Molecular Probes) were applied to each distal nerve stump^{25,27} and covered with a piece of gelatin sponge (Abgel, Sri Gopal Labs) to avoid spreading of the dye. The spinosus nerve was cut at the entrance into the skull to avoid retrograde diffusion of the tracer and contamination through extracranial branches of the mandibular nerve.

Postmortem Neuronal Tracing in Human and Rat Skulls.—Carbocyanine dyes like DiI are highly lipophilic, and therefore diffuse even in fixed tissues readily into and along the cell membrane of nerve fibers resulting in specific neuronal tracing.^{26,28} Therefore, they can be applied also at sites *ex vivo*, which are inaccessible *in vivo*. DiI shows a red fluorescence with the tetramethyl-rhodamine isothiocyanate (TRITC) filter and a pale green fluorescence with the fluorescein isothiocyanate (FITC) filter.²⁹

Immediately after placing the tracer, the skull halves of both species were fixed by immersion in 4% PFA dissolved in 0.1 M PBS buffer (pH 7.4) and incubated at 37°C in the dark for 6-7 months according to the estimated transport rate of 100-400 μ m/day with a diffusion coefficient of 107 cm²/s.³⁰ The labeled whole mounts were examined with a fluorescence stereomicroscope (MZ FL III, Leica, Bensheim, Germany). Thereafter, the human and rat cranial dura mater, the periost, and the pericranial muscles were removed from the skull. In addition in rats, the trigeminal ganglion and the brainstem were removed for evaluating the retrograde tracings. The pericranial muscles, the trigeminal ganglia, and the brainstem were placed in phosphate-buffered saline (PBS, pH 7.4) containing 30% sucrose at 4°C for 24 hours, quickly deep-frozen and cut into $15-\mu m$ longitudinal sections using a cryostat (CM 3050 S, Leica). After removing the soft tissue, the rat skulls were cryoprotected through a 20% sucrose solution for 48 hours at 4°C. Then they were placed in a 0.2-M EDTA solution (pH 7.4) for 15 weeks to decalcify the bone; the solution was changed every week. The decalcified skulls were again placed in a 20% sucrose solution for 24 hours at 4°C, quickly deep-frozen and cut into $20-\mu m$ longitudinal sections using a cryostat. The tissues and sections were mounted onto poly-L-lysine-coated 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 a rhodamine filter (FSet43wf) for optical viewing. Images were obtained using a DPSS laser (561 nm wavelength) and the TRITC filter unit (566-670 nm), or an Argon laser (488 nm) and the FITC filter unit (493-555 nm), for analysis. Two dry objective lenses ($10 \times$ and $20 \times$ with numerical apertures of 0.3 and 0.8), two oil-immersion objective lenses ($20 \times$ and $60 \times$ with numerical apertures of 0.8 and 1.4), and a $40\times$ water objective lens (numerical aperture 1.3) were used. The number of image pixels varied between 2048×2048 and 512×512 pixels. Data were merged into a 12-bit RGB tif-file using the confocal assistant software ZEN 2010 (Carl Zeiss MicroImaging). Images of trigeminal ganglion sections were used to measure the diameter of stained cell bodies containing a visible nucleus; for ovalshaped perikarya, the small diameter was taken.

Electron Microscopic Examinations of Rat and Human Spinosus Nerve.—Electron microscopy was used to examine the composition of axons of the spinosus nerve in rats and humans. Five rats were transcardially perfused with 0.9% saline, followed by 2.5% glutaraldehyde in PBS, and the skull was prepared as for tracing. The proximal part of the spinosus nerve was resected and kept in the same glutaraldehyde fixative overnight at 4°C. In the three human skulls, small pieces of the proximal spinosus nerve were dissected at the site where the nerve joined the MMA. The nerve segments of both species were rinsed in phosphate buffer overnight (4°C) 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



Fig 2.—Anterograde Dil tracing of human spinosus nerve. (A) Fenestrated human skull showing the position of the micrographs (B-G). Through a cranial window the middle cranial fossa of the contralateral side is visible. The tracer (Dil) was applied to distal branches of the spinosus nerve. (B) Situs of the branched spinosus nerve (outlined by white dotted lines) in the dura mater of the middle cranial fossa running along a branch of the middle meningeal artery (MMA, outlined by black dotted lines). The red arrow points to a site of tracer (Dil) application. (C and D) Ramified spinosus nerve distal to the tracing sites in the dura mater. Arrowheads point to single terminal fibers branching off from the main bundles. (E) Labeled fiber bundle at a branch of the middle meningeal artery (not visible). The arrowhead points to the tip of a terminating single fiber. (F) Small distal fiber bundle entering the skull (white oval) along an emissary venous vessel. The course of the fiber bundle within the bone is outlined by white dotted lines. (G) Labeled nerve fibers traveling through the endomysium of the temporal muscle. Arrowheads point to single fibers.

(Roth, Karlsruhe, Germany). Ultrathin cross-sections were cut with an ultramicrotome (Reichert Jung, Ultracut E; München, Germany), placed on copper mesh grids, stained according to standard methods with uranyl acetate and lead citrate, and examined with a Zeiss EM 906 transmission electron microscope (LEO, Oberkochen, Germany). Micrographs were taken with a monochrome digital camera connected to the microscope. The rough diameter of nerve fibers, which in myelinated fibers includes the myelin sheath, was assessed on the micrographs. Profiles deviating from a circle were approximated to an oval, and the mean of the long and the short axis was taken as the diameter.

RESULTS

Postmortem Anterograde Tracing in Rat Skulls.-The main nerve supplying the middle cranial fossa, referred to as spinosus nerve in analogy to the human morphology, arises from the mandibular division of the trigeminal ganglion. After anterograde staining near the trigeminal ganglion, the course of this nerve could be followed up to the most distal branches. Although there is some variability regarding the pattern of innervation, including the rare possibility of more than one nerve resembling the spinosus nerve, the most typical pattern is outlined in the following. The spinosus nerve runs initially along the occipital ankle of the middle cranial fossa, crosses the MMA, and divides into four to five main branches (Fig. 1B). One of these nerve branches runs toward the petrosquamous fissure and divides into two smaller branches. All other branches of the spinosus nerve run in parietal or rostral directions along the MMA. (Fig. 1B-D) These nerve bundles divide into smaller bundles, some of which cross and some accompany the arterial branches. In the whole course of the arborized spinosus nerve, some nerve fibers sheer out of the bundles and run into the adventitia of the MMA. Other branches leave the nerve bundles to run into the dural connective tissue and arborize dichotomously before they terminate (Fig. 1D). Following this pattern of innervation, the whole parietal and temporal dura is supplied by a dense network of nerve fibers. The innervation subserved by the spinosus nerve is restricted to the middle cranial fossa, ie, stained nerve fibers were

not seen in the superior sagittal sinus, the transverse sinus, or the cerebellar tentorium, nor in the frontal or occipital cranial fossa.

Along their way through the middle cranial fossa, stained bundles of nerve fibers were observed running into the cranial bone, where they enter particularly the intracranial openings of the emissary venules, and the sutures between the parietal and the frontal bone, the temporal and the parietal bone, and the temporal and the occipital bone (Fig. 1E,F). One of the main branches of the spinosus nerve, which entered the fibrous petrosquamous fissure, divided generally into two smaller bundles. In horizontal sections, the labeled nerve fiber bundles in the sutures and emissary canals were seen running transversally through the calvarium, while single fibers branch off and terminate in the diploe. After dividing into two branches inside the petrosquamous fissure, the nerve fiber bundles either cross the calvarium through the lambdoid suture, or run within the bone in occipital direction or along the posterior facial and the internal maxillary vein in caudal direction (Fig. 1F).

The retrogradely stained nerve fiber bundles were found leaving the cranial bone through the sutures and along the emissary veins cranially and caudally to the supramastoid crest of the temporal bone. The extracranial projection of nerve fibers is variable but follows a basic pattern. The bundles in the petrosquamous fissure leave the skull either through the lambdoid suture between the temporal and the occipital bone, or after following a longer course within the bone along the nuchal crest of the occipital bone. The fiber bundles enter the extracranial periosteum and run in close vicinity to arterial blood vessels, give off small branches and form a periostal network of trigeminal fibers similar to that of the dura mater (Fig. 1G). Finally, most of these nerve fibers transverse the periost and enter the tendinous junctions of the pericranial muscles. An especially dense innervation was found in the attachments of the upper nuchal muscles, ie, the splenius and longissimus capitis muscle, after the nerve fibers have left the skull along the nuchal crest between temporal and occipital bone. Some stained trigeminal fibers were found spreading deeply into connective tissue compartments of the pericranial muscles and even between muscle fibers (Fig. 1H,I). Surprisingly, a considerable proportion of the nerve fibers leaving the skull caudally to the supramastoid crest were found in the capsule of the temporomandibular joint (not shown). Stained nerve fibers were not seen above or in the vicinity of the sagittal suture, fitting the intracranial pattern of the innervation which is restricted to the middle cranial fossa. Inspection of the mandibular branch of the trigeminal nerve that leaves the skull did neither show any stained nerve fibers, and no labeling was observed in the greater or lesser occipital nerve. Furthermore, careful examination of the superior cervical ganglion provided no evidence of labeled nerve fibers or neurons.

Postmortem Anterograde Tracing in Human Skulls.—Using DiI placed to distal nerves dissected in the dura mater of the human skulls, we reinvestigated the course of meningeal fibers innervating the middle cranial fossa, comparable to our findings in the rat skull. Thereby, we confirmed the old observations of von Luschka, as well as of Penfield and McNaughton, and Dowgjallo^{2,3,5} on the course of the spinosus nerve, the main meningeal branch originating from the third (mandibular) division of the fifth cranial nerve. This nerve splits up into bundles, which run within the dura mater along the MMA and give rise to richly ramified endings (Fig. 2B-E). In addition, again confirming former findings and comparable to the anterograde tracings in rats, some of these bundles obviously penetrate the skull through the sutures and along the emissary veins (Fig. 2F). From our tracings, we estimate that about 10-20% of meningeal fibers of the spinosus nerve leave the skull in this way forming about 10 bundles on each side with myelinated and unmyelinated axons (see below). The stereomicroscopic observations showed regularly small nerve fibers bundles that sheer out of the spinosus nerve, follow the pars squamosa of the temporal bone, and penetrate the petrosquamos fissure. After the application of Dil crystals close to the penetration sites, we found traced fiber bundles on the outside of the squamous suture, and these bundles entered not only the periost but also the insertion of the temporal muscle (Fig. 2G). Due to the size of the human skull, it was not possible to trace the spinosus nerve arising from the mandibular division along its entire course.

Postmortem Retrograde Tracing in Rats.—Dil crystals were also placed to the proximal stump of the cut spinosus nerve near the trigeminal ganglion to stain the nerve fibers retrogradely. The cell bodies of these fibers were found in the maxillary and mandibular divisions of the ganglion (Fig. 3B). The peripheral axons of these neurons form 4-5 small nerve bundles running in the dura mater of the trigeminal ganglion (trigeminal cavum) along the mandibular nerve. Before the mandibular nerve leaves the skull base through the oval foramen, these nerve bundles leave the dura mater of the ganglion to enter the dura mater of the middle cranial fossa where they unite and form the spinosus nerve. Apart from the nerve fibers originating from their somata in the trigeminal ganglion, 40-50 axons were observed to pass the ganglion and the trigeminal nerve without any contact to cell bodies. The number of stained somata per ganglion ranged from 291 to 326 (mean \pm SD: 308.4 \pm 8.8; n = 25 ganglia), about 70% of which were located posteriolaterally the mandibular division within and 30% anterolaterally in the maxillary division (Fig. 3B-D). In the (anteromedially located) ophthalmic part, no labeled cell bodies were found. The diameter of stained somata ranged from 10 to $45 \,\mu\text{m}$, 65% of them showed diameters between 25 and $35 \,\mu m$ (Fig. 3D).

The central fibers of the pseudounipolar trigeminal ganglion cells leave the ganglion as a tight bundle and follow the spinal trigeminal tract in caudal direction (Fig. 3B). Labeled endings stained by DiI were found in the ipsilateral spinal trigeminal tract and in the superficial layers of the spinal trigeminal nucleus (Fig. 3E-G). No labeled fibers and neurons were detected on the contralateral side.

Electron Microscopic Examinations.—Crosssections through the proximal spinosus nerve were examined by electron microscopy in five rat and three human specimens, exhibiting remarkable similarities. The number of myelinated nerve fibers ranged from 156 to 162 in rat and from 165 to 171 in human spinosus nerves. Unmyelinated axons (ranging from 312 to 332 in rat and about 400 to 460 in human) were composed of 27-31 (rat) and about 25-30 (human) Remak bundles. The diameters of the myelinated



Fig 3.—Retrograde DiI tracing of rat spinosus nerve. (A) Hemi-schematic drawing of the rat brain showing the position of micrographs (B-C) and (E-G). The cerebral cortex is translucent to demonstrate the trigeminal ganglion with its nerves V1–3. The course of the spinosus nerve (N. spinosus) in the dura mater overlying the temporal and parietal cortex is indicated. (B) Whole mount fluorescent micrograph of the trigeminal ganglion. Labeled neurons are visible in the maxillary (V2) and mostly in the mandibular division (V3). Arrowheads point to labeled nerves traveling through the dura mater of the trigeminal ganglion, which unite and give rise to the spinosus nerve outside of the ganglion. The arrow indicates the course of the centrally running axons. (C) Labeled neurons in V3 at higher magnification in a trigeminal ganglion section. (D) Number of labeled neurons and distribution of soma diameters in trigeminal ganglia (mean \pm SD; n = 25). (E-G) Labeled centrally running axons in sections of the trigeminal tract at the entrance of the trigeminal root into the brainstem (E), at the transition between subnucleus oralis and interpolaris (F), and at the level of the subnucleus caudalis (G) of the spinal trigeminal nucleus.

axons (including myelin sheath) ranged from 1 to $6 \,\mu\text{m}$ and that of unmyelinated axons from 0.1 to 0.4 μm in the rat spinosus nerve (Fig. 4A-E). The diameter of axons in the Remak bundles of the human spinosus nerve could not reliably been assessed due to their limited conservation after immersion fixation (Fig. 4F-H).

Nerve fiber bundles leaving the rat skull through sutures and emissary canals (n = 11) contained typically few myelinated axons (mean \pm SD: 2.7 \pm 1.9) and considerably more unmyelinated axons (mean \pm SD: 15.2 \pm 1.1) (Fig. 4I,K). In addition, a distal branch of the spinosus nerve splits regularly up into two bundles containing around 30 myelinated and 60 unmyelinated fibers that enter the petrosquamous fissure.

DISCUSSION

The present neuronal *ex vivo* tracing study is complementary to our published *in vivo* tracing study combined with functional measurements.²⁴ In this work, we have described meningeal nerve fibers that spread through the skull and innervate extracranial tissues. This new concept was now confirmed by the present comparative study, which includes human tissue and allows reliable and complete tracing of



Fig 4.—Electron microscopy of the rat (A-E; I-K) and human spinosus nerve bundles (F-H). (A-B) Number of myelinated (A) and unmyelinated axons (B) and distribution of their diameters in the rat spinosus nerve (mean \pm SD; n = 5). (C-E) Cross-sections through the rat spinosus nerve. Remak bundles with unmyelinated axons are clearly visible at high magnification (arrow in E). (F-H) Cross-sections through the human spinosus nerve. Most myelinated and unmyelinated fibers in Remak bundles (arrows) show signs of degeneration. (I-K) Cross-sections through nerve fiber bundles found in the dura mater invaginating sutures of the rat skull.

nerve fibers. Using the antero- and retrograde *in vitro* tracing method in rats, we could demonstrate in detail the extended network of nerve fibers supplying the dura mater of the middle cranial fossa and adjacent extracranial structures. In addition, we examined the origin of trigeminal fibers in the trigeminal ganglion and their projection into the central nervous system. The observation of retrogradely labeled cell bodies in the trigeminal ganglion after application of the tracer to the same site of the spinosus nerve as for antero-

grade tracing confirms the conclusion that the nerve fibers identified intra- and extracranially after anterograde tracing belong to the trigeminal network of afferents that pass the dura mater. Preliminary tracings of other meningeal nerves reveal that the dura mater of the anterior and posterior cranial fossae is similarly innervated by nerve fibers that also give rise to extracranial projections. The precise innervation pattern of these areas will be examined in further studies. Afferent fibers innervating pericranial muscles through extracranial routes or motor efferents of the trigeminal nerve are unlikely to be among the labeled fibers because careful inspection of the mandibular branch that leaves the skull did not show any stained nerve fibers. Double labeling of neurons in the ganglion from the muscle and the dura mater using *in vivo* tracing techniques could ultimately confirm the concept of afferent collaterals innervating both tissues.

Of particular importance is that in both species, not only the periost but also the tendinous insertions and deep layers of pericranial muscles were found to be innervated by meningeal nerve fibers that transverse the cranial bone. The results will contribute to the understanding of the pathogenesis of primary and secondary headaches, and revive the discussion about the origin of these types of headaches.

Innervation of the Dura Mater of the Middle Cranial Fossa.—The application of DiI to the proximal spinosus nerve enabled the staining of all meningeal nerve fibers with all their ramifications up to the very terminals. Compared with the *in vivo* tracing method, this technique allows specific labeling of small regions of interest, eg, of one particular nerve.^{26,28} One disadvantage is that this type of labeling cannot be combined with immunohistochemistry.

The finding of a close relationship between the branches of the MMA and meningeal nerve fibers confirms previous classical histological^{3,5} and more recent immunohistochemical studies.^{10,12,20} Some nerve fibers of the nerves accompanying the arterial branches terminate in or close to the adventitia, but in the majority of cases small bundles of axons and single fibers sheer out of the main nerve, divide several times dichotomously, and extend with their terminals into connective tissue. We cannot exclude that nerve fibers running with or parallel to the spinosus nerve have a sympathetic^{31,32} or parasympathetic origin¹⁰ and contribute to vascular functions.^{33,34} However, the labeled axons are probably all afferent, since the spinosus nerve arising directly from the trigeminal ganglion was labeled at its most proximal end, and there is no evidence that the tracer can cross over to neighboring axons. The myelinated fibers identified in the electron microscopic cross-sections of the spinosus nerve are certainly trigeminal. The unmyelinated fibers running in Remak bundles are also likely to be afferent in nature because they are readily separated one from another by Schwann cell extensions (see Fig. 4E), which has been found to be a criterion for afferent fibers.³⁵

The innervation of the cranial dura mater by the ramifying spinosus nerve was restricted to the middle cranial cavity, and spared the sagittal and transverse sinus as well as the tentorium cerebelli. This confirms earlier findings of Strassman et al,¹² who defined two separated systems of meningeal afferent innervation: The meningeal structures surrounding the middle cranial fossa, sagittal and transverse sinus and tentorium, are supplied by a separate afferent pathway.

Transition of Nerve Fibers Through the Calvarium.-The penetration of meningeal nerve fibers into the calvarium along sutures and emissary canals and the innervation of the cranial bone have previously been demonstrated in the mouse by histochemical preparations.²³ Our anterograde tracings starting from the proximal spinosus nerve together with the retrograde tracings in rats, which stained somata in the trigeminal ganglion, show almost certainly that these bone penetrating fibers are of trigeminal origin. This is further confirmed by our recent functional studies on skull penetrating collaterals, which could be activated by noxious stimuli from the intracranial and the extracranial side, ie, from the dura mater and periosteum.²⁴ In the present study, we show the innervation pattern of these extracranially projecting afferents in more detail. We found labeled nerve fibers not only within the deep structures of the masticatory muscles and upper neck muscles but also within the connective tissue of the temporomandibular joint. Since we have never seen stained structures other than nerves in the dura mater, as well as in sutures and emissary canals, we are sure that the tracer did not freely diffuse to these extracranial tissues. In the nuchal region, the trigeminal innervation territory seems to overlap with the territory of the occipital nerves. The innervation of pericranial muscles by collaterals of meningeal afferent fibers seems to be fairly substantial. The labeled extracranial nerve fibers in this region are certainly not of spinal origin because staining was completely lacking in the occipital nerves. Although we observed, both in humans and rats, nerves fibers in the posterior part of the cranial cavity that penetrate the petrosquamous fissure, we could confirm labeled nerve fibers in the upper neck muscles only in rats, due to the limited diffusion distance of the postmortem tracing technique.

Role for Myelinated and Unmyelinated Fibers of the Spinosus Nerve.—The electron microscopic sections of the spinosus nerve both in rat and human specimens showed numerous myelinated nerve fibers, which according to their diameter must partly be classified as AB-fibers, confirming previous observations.9,12,20 Aβ-fibers subserve normally mechanoreceptive functions, but it seems difficult to attribute meningeal afferents a non-nociceptive function since there is no sensation but pain that could be evoked by stimulation of the dura mater during head surgeries.^{5,6} It has been speculated that these nerve fibers could be activated by mechanical stimuli, such as sudden head movements.^{12,36} This idea is particularly interesting in respect of their possible contribution to migraine and chronic tension-type headaches, if these nerve fibers belong to those that innervate pericranial muscles. To clarify the nociceptive nature of these afferents, combined labeling with nociceptive markers like neuropeptides may be useful, but first trials using antibodies against calcitonin gene-related peptide to label retrogradely traced nerve fibers have failed, probably due to the long duration of tissue fixation.

Apart from the above hypothesis, there is an additional functional explanation for the extracranial innervation: Possibly part of the myelinated nerve fibers innervating pericranial muscles are not collaterals of meningeal afferents but proprioceptive fibers that travel through the trigeminal ganglion toward their somata located in the mesencephalic nucleus of the trigeminal nerve.³⁷ Those fibers may preferably be myelinated, and indeed the small distal nerve bundles approaching the sites of skull penetration seem to contain a higher proportion of myelinated vs unmyelinated fibers compared with the proximal spinosus nerve, particularly bundles that penetrate the petrosquamous fissure – compare figure 1e in Schueler et al²⁴ with Figure 4 of the

present paper. The size distribution of neuronal cell bodies, which may approximately reflect the diameters of axons, seems to be in accordance with the above distribution in that the ratio of small cells (below 20 μ m) labeled by *in vivo* tracing from the periosteum in our previous examination is lower than the ratio of small neurons labeled by *ex vivo* tracing of the spinosus nerve – compare figure 2d of Schueler et al²⁴ with Figure 3D of the present paper.

Whereas the role for $A\beta$ -fibers in meningeal nociception is unclear, there is good reason to assume that the skull penetrating, presumably nociceptive, $A\delta$ and C-fibers are involved in the generation of headaches. This pattern of innervation could, for example, explain the aggravating influences of neck muscle tension on tension-type headache and migraine,^{38,39} and may explain why manual therapies of pericranial structures can be successful in the management of headaches.⁴⁰ It may also partly be an explanation for the beneficial effects of local anesthetic or botulinum toxin injections into peripheral nerves, or the so-called trigger points of pericranial tissues.^{41,42}

Trigeminal Ganglion and Central Projections.— The dominance of small labeled cell bodies in the trigeminal ganglion is in accordance with the dominant number of unmyelinated axons counted in the electron micrographs of the cross-sected spinosus nerve. The cell bodies of the retrogradely labeled trigeminal fibers of the spinosus nerve were found exclusively within the maxillary and mandibular divisions of the trigeminal ganglion, ie, more than 70% of neurons were located in the posterolateral part of the mandibular division. This surprising result is in accordance with our recent study²⁴ but is not consistent with previous in vivo studies that show an ophthalmic contribution.^{36,43} The most likely reason for this discrepancy between the present and the above studies is the application site of the tracer to the dural tissue around the MMA and near the superior sagittal sinus, areas that seem to be innervated by neurons both from the mandibular and the ophthalmic division. Strassman et al (2004),¹² using DiI application in formalin-fixed tissue, described two separate systems of nerve fibers in the dura mater, one that runs parallel to the MMA and another with a

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preferentially orthogonal orientation running from the transverse sinus across the MMA. The latter may arise from tentorial nerve fibers, which origin in the ophthalmic division of the ganglion. In contrast, the present postmortem anterograde tracings enabled the selective application of the tracer to the spinosus nerve, exclusively innervating the dura mater of the middle cranial fossa.

The central extension of the retrogradely labeled nerve fibers project through the spinal trigeminal tract into the spinal trigeminal nucleus, which is in accordance with previous tracing studies. Labeled nerve fibers innervating cranial blood vessels, either intracranial (MMA, superior sagittal sinus) or extracranial (superficial temporal artery), were found to terminate centrally in the interpolar and caudal subnucleus of the spinal trigeminal nucleus.^{36,44,45} The spinal trigeminal nucleus is known to be mainly involved in the transmission of nociceptive information from inside and outside the head and the face.⁴⁶⁻⁴⁸ A spatial separation of intra- and extracranial nociceptive transmission has not been identified, which underlines the idea that both intra- and extracranial afferent input can contribute to the generation of headaches.

Taken together, we conclude from our comparative tracing study in the rat and human skull that, due to the high homology of the trigeminal innervation, the rat is a valid model to study the anatomical and functional characteristics of the meningeal innervation with regard to pathophysiological aspects of head pain. The main conclusion drawn from this study is that the pericranial nociceptive innervation, which is partly arising from the intracranial meningeal innervation, may significantly contribute to the generation of headaches.

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STATEMENT OF AUTHORSHIP

Category 1

(a) Conception and Design

Karl Messlinger; Markus Schueler; Winfried L. Neuhuber

- (b) Acquisition of Data Markus Schueler; Karl Messlinger; Roberto De Col
- (c) Analysis and Interpretation of Data Markus Schueler; Karl Messlinger; Winfried L. Neuhuber

Category 2

(a) Drafting the Manuscript

Markus Schueler; Karl Messlinger

(b) Revising It for Intellectual Content Karl Messlinger; Roberto De Col; Winfried L. Neuhuber

Category 3

(a) Final Approval of the Completed Manuscript

Markus Schueler; Karl Messlinger; Roberto De Col; Winfried L. Neuhuber

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