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Sensory innervation of the calvarial bones of the mouse

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Abstract

Migraine sufferers frequently testify that their headache feels as if the calvarial bones are deformed, crushed, or broken (Jakubowski et al., 2006). This has lead us to postulate that nociceptive fibers supply the calvarial bones. We studied sensory innervation of the calvaria in coronal and horizontal sections of whole-head preparations of postnatal and adult mice, using immunostaining of peripherin – a marker of thinly-myelinated and unmyelinated fibers – and calcitonin gene-related peptide (CGRP) – a marker more typical of unmyelinated nerve fibers. In pups, we observed nerve bundles coursing between the galea aponeurotica and the periosteum; between the periosteum and the bone; between the bone and the meninges; as well as fibers that run inside the diploë in different orientations. Some dural fibers issued collateral branches to the pia at the frontal part of the brain. In the adult calvaria, the highest concentration of peripherin- and CGRP-labeled fibers were found in sutures where they appeared to emerge from the dura. Labeled fibers were also observed in emissary canals, bone marrow and periosteum. In contrast to pups, no labeled fibers were found in the diploë of the adult calvaria. Meningeal nerves that infiltrate the periosteum through the calvarial sutures may be positioned to mediate migraine headache triggered by pathophysiology of extracranial tissues, such as muscle tenderness and mild trauma to the skull. In view of the concentration of sensory fibers in the sutures, it may be useful to avoid drilling the sutures in patients undergoing craniotomies for a variety of neurosurgical procedures.

Keywords

migraine; headache; pain; trigeminal; CGRP

INTRODUCTION

Acute or chronic headache ensuing from cranial neurosurgery (craniotomy), as well as non-surgical migraine headache, are commonly considered in terms of nociceptive innervation of the dura or pericranial muscle and soft tissue (de Gray and Matta, 2005; Olesen et al., 2005; Penfield and McNaughton, 1940) rather than bone itself. Yet, migraine sufferers frequently testify that their headache feels as if the calvarial bones are deformed, crushed, or broken (Jakubowski et al., 2006). Could this perception of pain originate in sensory nerve fibers inside the bones of the calvaria?

Bones of the calvaria are abutted by intracranial (endosteal) and extracranial (periosteal) sheaths that are richly-supplied by sensory, sympathetic, and parasympathetic innervation (Alberius and Skagerberg, 1990; Herskovits et al., 1993; Hill and Elde, 1991; Kruger et al.,

1989; Silverman and Kruger, 1989). While the endosteum and periosteum are innervated by nociceptors originating in the dura mater and scalp tissues, respectively, little is known about the innervation of the diploë – the osseous part of the calvaria. In neonatal rats, bundles of adrenergic fibers in the dura were found to branch into the endosteum and traverse the diploë (Alberius and Skagerberg, 1990). In weanling rats, postganglionic fibers originating in the superior cervical sympathetic ganglia were found to terminate in endosteum, periosteum, and diploë, mostly in association with blood vessels (Herskovits et al., 1993). In both of these studies, the calvarial sutures (sagittal, coronal, or metopic) were conspicuously devoid of sympathetic innervation. In adult rats, the calvarial periosteum and diploë were found to be innervated by sympathetic fibers immunoreactive to vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), or dopamine- β -hydroxylase, and by an extensive network of sensory fibers immunoreactive to calcitonin gene-related peptide (CGRP) or substance P (Hill and Elde, 1991). Since capsaicin pretreatment greatly reduced the immunostaining for CGRP and substance P, it was concluded that the calvarial periosteum and diploë are richly supplied by nociceptors (Hill and Elde, 1991).

To examine whether the membranous bones of the skull contain nerve fibers that are consistent with sensory innervation, we used whole-head preparation of mouse pups and decalcified whole-head preparation of adult mice. Detection of nerve fibers was performed using two immunohistochemical markers of peripheral nerves: peripherin which is present in thinly-myelinated and unmyelinated fibers (Garry et al., 2005; Goldstein et al., 1991), and calcitonin gene-related peptide (CGRP), which is more typical of unmyelinated nerve fibers (Alvarez et al., 1991; Caterina et al., 1997; Goldstein et al., 1991; Guo et al., 1999; Ichikawa et al., 2002).

MATERIALS AND METHODS

Animals

Experiments were approved by the Standing Committee on Animals at Harvard Medical School. Male and female C57Bl/6J and BALB/cJ mice were sacrificed at an early stage of development (2-7 days of age) or adulthood (4-6 months of age). Some pups were sacrificed using an overdose of Avertin (1 g/kg body weight, i.p.); their heads were removed and immediately frozen on dry ice to be stored at -20°C . Other pups, as well as adult mice, were perfused transcardially either with 4% paraformaldehyde in 0.1 M phosphate buffered saline solution (PBS; pH 7.4) or with Zamboni's fixative (2% paraformaldehyde, 0.2% saturated picric acid, in PBS) under deep Avertin anesthesia (0.25 g/kg body weight, i.p.). Heads were removed and stored overnight at 4°C in a fresh fixative solution as the one used for perfusion, then cryoprotected through 20% and 30% sucrose solutions at 4°C . Adult heads were immersed at 4°C over 10 weeks in 0.2 M EDTA, pH 7.4 (with changes for fresh solution every other day) in order to decalcify the bones of the skull. Decalcified heads were cryoprotected using sucrose solution as described above.

Tissue processing

Heads were equilibrated to -18°C in the cryostat chamber (Leica) and cut into 30- μm -thick sections. Coronal or horizontal sections were collected serially, thaw-mounted onto gelatin-coated glass slides (3-8 sections/slide), and stored at -20°C . Sections were thawed up to room temperature on the day 1 of immunohistochemistry. Sections from non-perfused pup heads were fixed for 30 min in PBS containing 4% paraformaldehyde. All sequential sections from each head preparation were processed with only one primary antibody.

Primary antibodies

All sections were washed through 3 changes of PBS, 10 min each. The peripherin primary antibody was a polyclonal rabbit antiserum raised against electrophoretically pure trp-E-peripherin fusion protein containing all but the four N-terminal amino acids of rat peripherin (Chemicon, Temecula, CA, AB1530 diluted 1:5,000). According to the manufacturer, Western blot analysis of cytoskeletal extract from the rat spinal cord specifically stains ~57 kDa band (Errante et al., 1994). The CGRP primary antibody was a polyclonal rabbit antiserum against an antigen corresponding to amino acids 83-119 of the rat and mouse α -CGRP (Bachem, T-4032 diluted 1:10,000). This antibody was characterized using Western blot analysis and immunohistochemistry as shown in Fig. 1. Twenty-five μ g of total protein from homogenized trigeminal ganglia and 1 ng of purified α -CGRP (Bachem) were electrophoresed through 16.5% SDS-polyacrylamide Tricine gel (BioRad). The electrophoresed material was transferred to PVDF membrane (Millipore) using wet electroblotting at 30 V overnight. For control, the CGRP antibody (1:10,000) was preadsorbed for 2 h with access amount of purified α -CGRP.

All antibody dilutions used in immunohistochemistry were made using a blocking solution (2% goat serum; 2% fetal bovine serum; 0.5% Triton X-100; 0.1 M PBS).

Secondary antibodies

For fluorescence microscopy, we used goat anti-rabbit secondary antibody conjugated to fluorescent isothiocyanate (FITC). For transmitted-light microscopy, we used a biotinylated goat anti-rabbit secondary antibody. Secondary antibodies were obtained from Jackson Immunolabs (West Grove, PA) and were diluted 1:1,000 in blocking solution.

Immunofluorescence

To minimize nonspecific binding of the primary antibodies, sections were pre-incubated with the blocking solution (50 μ l/section) for 2 h at room temperature. After blotting off the blocking solution, sections were incubated overnight at 4 °C with the designated primary antibody solution (50 μ l/section). Sections were then washed 3 $\times$ 10 min in PBS, incubated for 2 h at room temperature with the designated secondary antibody-FITC conjugate solution (50 μ l/section), and washed again 3 $\times$ 10 min in PBS. Finally, sections were cover-slipped using Vectashield mounting medium (Vector Labs, Burlingame, CA).

Immunodetection with transmitted light

To minimize endogenous peroxidase activity, sections were pre-incubated with 0.3% H₂O₂ in PBS for 30 minutes at room temperature and washed in PBS 3 $\times$ 10 min. Subsequent steps were performed as described above, including incubations with blocking solution, primary antibody, secondary antibody, and standard PBS washes. To detect the immunoreactive product, sections were incubated with an ABC solution for 1 h at room temperature, washed 3 $\times$ 10 min in PBS, and incubated with diaminobenzidine (DAB) solution for 2-8 min at room temperature according to the vendor recommendation (Vector Labs). Finally, sections were washed 3 $\times$ 10 min in PBS, dehydrated through increasing alcohol concentrations (50-100%), cleared in xylene and cover-slipped using Cytoseal 60 mounting medium (Richard-Allen Scientific, Kalamazoo, MI).

Photomicrography

) to be viewed and imaged using fluorescent-light microscopy (Olympus) and digital photography (Cool Snap).

Digital photomicrographs were adjusted in Adobe Photoshop software to optimize the histograms without clipping using the Levels tool, and to convert from RGB mode to grayscale.

Methods section that describes the details of your photomicrograph production, specifying the software used to store/manipulate your photomicrographs (such as Photoshop or Illustrator) and any modifications (such as to contrast or brightness) that were done.

RESULTS

Calvarial nerve fibers immunoreactive to peripherin or CGRP were studied using coronal (Fig. 2A) and horizontal (Fig. 2B) sections collected serially from a whole-head preparation of pups and adult mice. The relative specificity of the antibodies was first confirmed on the basis of the size of perikarya immunolabeled in the trigeminal ganglion. Peripherin immunostaining was present mostly in medium- and small-size perikarya (Fig. 2C, D). CGRP immunostaining labeled mostly small-size perikarya (Fig. 2E, F). Innervation of calvarial bone marrow cavities, emissary canals, sutures, periosteum, and the leptomeninges (Fig. 2G) will be described below. Peripherin is more prevalent in sensory fibers than in motor and autonomic fibers (Escurat et al., 1990; Silverman and Kruger, 1989). CGRP is more prevalent in nociceptive fibers than motor or autonomic fibers (Kruger et al., 1989; Silverman and Kruger, 1989).

Calvarial innervation in mouse pups

In the pup calvaria, nerve fibers immunoreactive to peripherin and CGRP traversed (directionality undetermined) the calvarial bone between the periosteum and dura, through the diploë and endosteum, at multiple locations in frontal, parietal and interparietal areas of the head (Fig. 3; photo insets in Fig. 4). Camera lucida tracing of peripherin-labeled fibers from 6 consecutive coronal (Fig. 4A) and horizontal (Fig. 4B) sections of the head showed dense innervation of calvarial bones at the roof of the skull. At the coronal plan, fibers crossing the bone were distributed evenly throughout the bone (Fig. 4A). At the horizontal plan, densities of fibers crossing the frontal, parietal and interparietal bones were higher in areas analogous to the human forehead, temples, and the back of the head (Fig. 4B).

Calvarial innervation in the adult mouse

In the adult calvaria, peripherin- and CGRP-labeled fibers were observed in the bone marrow (Fig. 5), emissary canals (Fig. 6), and sutures (Figs 7-8), but not in the diploë. Fibers that entered the emissary canals and bone marrow cavities branched out of periosteal and dural nerves; fibers that entered the sutures branched out of dural nerves (Fig. 6).

Bone marrow—Labeled nerve bundles were observed running and branching in calvarial bone marrow cavities in a variety of orientations: anterior-posterior; dorsoventral; mediolateral. Within the thickness of the section it was not possible to follow the entire length of individual fibers. In few cases, fibers were seen extending between bone marrow and sutures (Fig. 5A) and between bone marrow and emissary canals (Fig. 6C, D). Bone marrow innervation derived from emissary canals that opened extracranially to the periosteum (Fig. 6B, E) and emissary canals that opened intracranially to the dura (Fig. 6C, D). Both peripherin- and CGRP-positive fibers were seen at close proximity to bone marrow cells (Fig. 5E-G) whose morphology was consistent with red blood cells, leucocytes, megakaryocytes, osteoblasts, and capillary endothelial cells.

Emissary canals—Emissary canals are passages in bones of the calvaria through which emissary veins drain blood from dural sinuses and diploic veins of the bone. In the emissary canals we found peripherin- and CGRP-labeled fibers that originate in either extracranial nerve bundles that run along the periosteum (Fig. 6B, E), or intracranial bundles that run along the dura (Fig. 6C, D). Emissary canals that crossed the diploë uninterrupted between periosteum and endosteum appeared to provide a direct passage of innervation between scalp and dura (Fig. 6A), but the directionality of such fibers could not be determined.

Cranial vault sutures—The calvarial sutures consists of fibrous tissue that serves as an area of intramembranous bone growth during development, and marks the borders of the matured bones in adulthood. We found that calvarial sutures constitute major passageways through which peripherin- and CGRP-labeled fibers establish connection between the meninges and scalp (Figs. 7-14). The mass of labeled fibers followed the very complex architectural organization of the calvarial sutures including the sagittal (Figs. 7, 9-11), coronal (Figs. 8, 10, 12, 13), squamos (Figs. 10, 11, 14) and lambdoid (Figs. 12-13).

Distribution of peripherin-positive fibers – coronal plane (Figs. 9-11)—Tracing of peripherin-labeled fibers from consecutive coronal sections of the head showed (i) dense innervation of the sagittal sinus at rostral and caudal end of the skull and sparse innervation in between; (ii) abundant labeled fibers in the coronal and squamos sutures and in the emissary canals; and (iii) virtually no fibers in the diploë. The paucity of labeled fibers in the bone marrow may be due to loss of marrow content during tissue processing.

Distribution of CGRP-positive fibers – horizontal plane (Figs. 12-14)—Tracing of CGRP-labeled fibers from consecutive horizontal sections of the head showed (i) dense innervation of the coronal and squamos sutures and appreciable innervation of the lambdoid suture; (ii) higher density of labeled fibers on the side of the suture facing the dura than on the side facing the periosteum; and (iii) no innervation of the diploë.

Taken together, mapping of peripherin- and CGRP-labeled fibers in coronal and horizontal section suggest that most nerve fibers are present in sutures at the dorsal aspect of the calvaria.

Origin and direction of calvarial nerve fibers—Points of entry into the sutures were demonstrated by bundles of nerves branching into the sutures from the dura (Fig. 15 A-D). On the periosteal side we did not find nerves that branched into the sutures; rather, we observed labeled fibers that extended between sutures and periosteum without apparent change in thickness (Fig. 15E, F), which would be consistent with fibers emerging from the sutures into the periosteum. These fibers, however, are not the main source of the rich web of peripherin- and CGRP-labeled fibers in the periosteum (Fig. 16A-C); the majority of periosteal nerves seem to derive from extracranial nerves in the galea aponeurotica (Fig. 17A, B).

Dura-pia collaterals (Fig. 18)—In the pups, dural CGRP-labeled fibers were observed issuing collateral branches that crossed the arachnoids and entered the pia (Fig. 18). In the adult, such branches were not preserved because the decalcification process resulted in a large gap between the dura and pia which tore away the arachnoids.

DISCUSSION

Using immunohistochemistry for peripherin and CGRP, we have documented the innervation of the calvaria in mouse pups (P2-P7) and adult mice. In the developing calvaria, nerve fibers were observed traversing the bone through periosteum, diploë, endosteum, dura, arachnoid and pia at multiple locations in no particular pattern. We were unable to determine whether fibers crossing the developing bones originated in the periosteum (i.e., traveling inward) or in the dura (i.e., traveling outward). In the mature calvaria, the diploë was all but devoid of nerve fibers; instead, the sutures appeared to constitute the major passageway of innervation between the dura and periosteum. These fibers appeared to enter the suture from the dura and emerge into the periosteum.

Cell soma of neurons that contain peripherin and issue axons outside the central nervous system are found in trigeminal ganglia (Goldstein et al., 1991; Lariviere et al., 2002; Potrebic et al., 2003), motor neurons of the ventral horn (Escurat et al., 1990), and autonomic postganglionic

neurons (Escurat et al., 1990). Cell soma of neurons that contain CGRP and issue axons outside the central nervous system are found in the trigeminal ganglion (McCulloch et al., 1986; O'Connor and van der Kooy, 1986; Potrebic et al., 2003; Stjarne et al., 1989; Tajti et al., 1999), brainstem motor neurons (de Souza et al., 2008; Escurat et al., 1990), and parasympathetic ganglion cells (Lee et al., 1985; Silverman and Kruger, 1989; Stone et al., 1988) containing vasoactive intestinal polypeptide and choline acetyltransferase (Hardebo et al., 1992; Suzuki et al., 1989). Interpretations of peripherin- and CGRP-positive fibers in periosteum, endosteum, sutures, emissary canals, and bone marrow of the calvaria must therefore take into consideration the possibility that the labeled fibers originate in motor, autonomic and sensory neurons.

Motor origin of labeled fibers in calvarial bones seems unlikely for two reasons. Functionally, sutures and bone cavities are devoid of smooth or skeletal muscles. Anatomically, the majority of labeled fibers in the calvarial bones stemmed from dural nerves; dural nerve fibers originate in sensory, sympathetic and parasympathetic ganglia but not motor neurons (Edvinsson et al., 2001; Edvinsson et al., 1989; Liu et al., 2004; 2008).

Sympathetic innervation of calvarial bones (Alberius and Skagerberg, 1990; Herskovits et al., 1993) and the meninges (Edvinsson et al., 2001; Edvinsson et al., 1989; Liu et al., 2004; 2008) is well documented. Such innervation originates in post-ganglionic neurons in the superior cervical ganglion whose fibers exhibit VIP, NPY, or dopamine- β -hydroxylase immunoreactivity. Since sympathetic ganglia do not contain CGRP perikarya (Silverman and Kruger, 1989), we conclude that the CGRP-positive fibers observed in the calvarial bones were not sympathetic.

Cranial parasympathetic ganglia (i.e., otic, ciliary, sphenopalatine, submandibular) contain perikarya immunoreactive to CGRP (Silverman and Kruger, 1989) and peripherin (Escurat et al., 1990; Silverman and Kruger, 1989). Functionally, parasympathetic innervation of emissary canals and bone marrow may regulate emissary veins that drain blood from the bone marrow and sinuses, as well as blood vessels supplying periosteum and endosteum. In agreement with a previous study (Hill and Elde, 1988), labeled fibers in these areas were often seen in association with blood vessels. However, capsaicin-treated animals exhibit marked reduction in the density of CGRP-positive fibers in the calvarial periosteum (Hill and Elde, 1988), and transection of the trigeminal nerve abolishes CGRP labeling in cerebral blood vessels and the dura (McCulloch et al., 1986). Since CGRP fibers in the sutures were found to arise from the dura, we propose that the sutural CGRP fibers represent mainly sensory innervation. Such sensory innervation is consistent with nociception since CGRP immunoreactivity is present mostly in small-size perikarya that give rise to unmyelinated axons (Alvarez et al., 1991; Caterina et al., 1997; Goldstein et al., 1991; Guo et al., 1999; Ichikawa et al., 2002).

CGRP innervation of bone marrow may be important for metabolism of bone cells, neuro-immune modulation of haematopoiesis, control of peripheral blood cell number, and modulation of colony forming cells (Tabarowski et al., 1996; Zaidi et al., 1988; Zaidi et al., 1987). Since CGRP-positive fibers could be nociceptors, their presence near red blood cells, leucocytes, megakaryocytes, osteoblasts, and capillary endothelial cells calls attention to the possibility that some of these cells, particularly the immune ones, play a role in the pathophysiology of some headaches. Under certain conditions and in response to certain stimuli, bone marrow nociceptors could be activated by leucocytes degranulation and the release of algogenic molecules such as superoxide, hydrogen peroxide, hydroxyl radicals, toxic nitrogen oxide, growth factors (e.g., TGF), cytokines (e.g., IL-1, 2, 4, 6; TNF- α) and lipid mediators such as prostaglandins and eicosanoids.

The apparent contribution of dural nerve fibers to branches that branch out into the sutures of the adult calvaria is consistent with a line of evidence that the subsutural dura gives rise to neural crest cells that differentiate into the osteoblasts that form the bones of the calvaria (Gakunga et al., 2000; Lenton et al., 2005; Opperman, 2000). It is tempting to propose that meningeal pain fibers penetrate the developing bone through the patent calvarial sutures and become integrated into the expanding bone, such that they can be observed crossing the bone through the diploë at multiple sites. However, the findings from the adult calvaria suggest that diploic nerve fibers eventually disappear from the diploë later in development.

Dural CGRP-labeled fibers were observed issuing collateral branches that crossed the arachnoids and entered the pia in the frontal part of the brain of the pup. Whether or not such connections exist in the adult calvaria could not be determined due to large gaps between the dura and leptomeninges in the adult head preparations, presumably due to shrinkage of the bone during the decalcification process which pulled the dura away from the leptomeninges. Available evidence in the literature does not allow us to determine whether individual trigeminal ganglion neurons issue sensory axons to both dura and leptomeninges, or whether their innervation is distinct (Liu et al., 2004; 2008; Uddman and Edvinsson, 1989; Uddman et al., 1989). Dura-pia connections is consistent with the hypothesis of a cross-talk between the two, and could potentially mediate plasma protein extravasation and neurogenic inflammation that develop in the dura following the induction of cortical spreading depression (Bolay et al., 2002; Iadecola, 2002). Our observation of CGRP fibers stretching directly between the dura and pia provides first anatomical evidence to support such neural interaction.

It has long been held that migraine headache is referred to the periorbital/temporal area by central nociceptive neurons that are rendered abnormally hyperactive by input from meningeal pain fibers (Penfield and McNaughton, 1940; Ray and Wolff, 1940). The present findings have lead us to suggest that migraine headache affecting the periorbital/temporal area may, in some cases, be driven locally by extracranial collaterals of meningeal pain fibers. Using the concept of local release of proinflammatory molecules from pain fibers that are activated antidromically by distant collaterals of the same nociceptor, we would like to propose two scenarios by which nociceptive fibers that project from the dura to extracranial tissues can mediate migraine pain.

Extracranial origin of intracranial pain (Fig. 19A)

In this scenario, action potentials generated at extracranial collaterals of meningeal pain fibers spread antidromically to collaterals that terminate inside the cranium, resulting in local release of proinflammatory neuropeptides and activation of neighboring meningeal nociceptors. This scenario may explain the induction of migraine headache by extracranial triggers, such as tenderness of scalp muscles, or minor head trauma affecting the periosteum.

Intracranial origin of extracranial pain (Fig. 19B)

In this scenario, action potentials generated intracranially at the leptomeningeal pain fibers spread antidromically to collaterals that terminate outside the cranium, resulting in activation of neighboring somatic nociceptors through local release of proinflammatory neuropeptides in both the dura and the scalp. This concept would be consistent with extracranial perivascular edema observed in some patients undergoing a migraine attack (Graham and Wolff, 1938; Wolff et al., 1953). This scenario may explain the perception of ‘imploding headache’ (i.e., perception of pain on the outside of the skull; Jakubowski et al., 2006) in migraine triggered by intracranial events, such as aura.

Finally, the presence of CGRP fibers in calvarial sutures, endosteum and periosteum in the present study may constitute a neural substrate for the perception of deformed, crushed, or broken skull during migraine (Jakubowski et al., 2006).

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Figure 1.

Western blot analysis (A-D) and immunohistochemistry (E-G) of CGRP in the mouse trigeminal ganglion. A, B, Immunoblot for CGRP using commercial α -CGRP, showing a band corresponding to the 37 amino-acid peptide (A), lysate of trigeminal ganglia showing bands corresponding to the CGRP peptide and the 128 amino-acid CGRP precursor (B). C, D, Same as A and B, except the CGRP antibody was preadsorbed with α -CGRP, showing no detectable material. Numbers indicate molecular marker size (kDa). E, Immunostaining of CGRP-positive cell bodies in the trigeminal ganglion. F, Same as E, except using preadsorbed CGRP primary antibody. G, Same as E, except no primary antibody was used. Scale bar for E-G: 100 μ m.

Figure 2.

Histological analysis of the skull and characterization of trigeminal ganglion perikarya immunostained for peripherin and CGRP. A, B, Schematic illustration of the bones of the calvaria showing 3 coronal (A) and 3 horizontal (B) planes of analysis (red lines). Shown below are whole head preparations corresponding to the frontal coronal plane (A) and the middle horizontal plane (B). Immunostaining specificity was based on the size of labeled perikarya in the trigeminal ganglion. Peripherin-labeled perikarya were mostly medium- and small-size in pups (C) and adults (D). CGRP-labeled perikarya were mostly small-size in pups (E) and adults (F). Arrows point to labeled perikarya. Arrows point to labeled perikarya. Scale bar: 100 μ m.

G, Nomenclature of the different layers of the scalp and skull. Sections in A, B, G were stained with hematoxylin and eosin.

Figure 3.

Nerve fibers traversing the calvarial bones in mouse pups. A, Peripherin-labeled fibers crossing the diploë of the parietal bone. B-D, confocal imaging of peripherin fibers that cross bones of the calvaria between the galea aponeurotica and meninges. B, normal view of a fiber stretching between the dura and bone. C, side view (a 90° lateral rotation of the image shown in B) demonstrating that the fiber continues its run across the bone and into the galea aponeurotica. Orientations of B and C are indicated by the box inserts, where the gray face of the box marks the top aspect of the tissue. D, side view resulting from a 45° vertical rotation of the image shown in B, demonstrating the entire course of the fiber between the dura and galea aponeurotica (the top face of the box corresponds to the gray faces of the boxes in B and C). E, F, examples of CGRP-labeled fibers crossing the diploë of the calvarial bones. Arrowheads point to labeled fibers. Scale bars: 50 μ m.

Figure 4.

Camera lucida reconstruction of peripherin nerve fibers in bones of the calvaria in the mouse pup. A, fibers in the parietal bone reconstructed from coronal sections of the head. B, fibers in the frontal, parietal and interparietal bones reconstructed from horizontal sections of the head. Note the concentration of labelled fibers in the frontal, supraorbital and caudal areas of the skull. Each drawing (A, B) represents cumulative information from 6 consecutive 30-μm-thick sections. Details in the boxed regions are shown in the corresponding photomicrographs.

Figure 5.

Bone marrow in the adult calvaria. A-D, peripherin-labelled fibers. E-G, CGRP-labelled fibers. All images are taken from horizontal sections. Arrowheads point to labelled fibers. Scale bar: A, C – 200 μ m; B, G – 100 μ m; D-F – 50 μ m.

Figure 6.

Emissary canals in the adult calvaria. A-D, peripherin-labelled fibers. E-F, CGRP-labelled fibers. A – coronal section; B-F – horizontal sections. Arrowheads point to labelled fibers. Scale bar: A, B, D, E – 100 μ m; C, F – 50 μ m.

Figure 7.

Sagittal suture in the adult calvaria. A, B, peripherin-labelled fibers running in the sagittal suture between the frontal (A) and the parietal (B) bones. C, CGRP-labelled fiber running in the sagittal suture between the frontal bones. All images were taken from coronal sections. Arrowheads point to labelled fibers. Scale bar: A, C – 50 μ m; B – 100 μ m.

Figure 8.

Nerve fibers in the coronal suture of the adult calvaria immunostained for peripherin (A-F) and CGRP (G-J). A, C, low-power views of the convoluted architecture of the suture. B, D, magnified views of the insets A and C, respectively. G-J, high-power images of multiple CGRP-labelled fibers that follow the curves of the suture. A-F, coronal sections; G-J, horizontal sections. Arrowheads follow labelled fibers. Scale bar: A, C – 200 μm ; B – 25 μm ; D-J – 50 μm .

Figure 9.

Coronal view of peripherin-labelled fibers in the sagittal suture at the rostral plane of the adult calvaria. At this plane of the olfactory bulbs (bottom illustration and photomicrograph), the sagittal suture constitutes a distinct passageway for nerve fibers between the meninges and periosteum, as illustrated in the camera lucida reconstructions on top. Reconstruction corresponding to the area marked by box in photomicrograph represents labeled fibers that were found in 84 consecutive sections, covering about 2.5 mm. Blue areas depict bone marrow. Red line indicates the plane of analysis. ob, olfactory bulbs.

Figure 10.

Coronal view of peripherin-labeled fibers in the coronal suture at the middle of the adult calvaria. At this plane of the motor cortex (bottom illustration and photomicrograph), the coronal suture contains fibers that run at rostrocaudal, dorsoventral and mediolateral orientations, as shown in the camera lucida illustrations on top. Reconstruction corresponding to the area marked by box in photomicrograph represents labeled fibers that were found in 99 consecutive sections, covering about 3.0 mm. Note absence of labeled fibers in the diploë. Blue areas depict bone marrow. Red line indicates the plane of analysis. Abbreviations: cc, corpus callosum; oc, optic chiasm; tg, trigeminal ganglion.

Figure 11.

Coronal view of peripherin-labeled fibers in the sagittal and squamos sutures at the caudal plane of the adult calvaria. At this plane of the visual cortex (bottom illustration and photomicrograph), the sutures define a continuum of nerve fibers extending between the dura and periosteum, as shown in the camera lucida illustrations on top. Reconstructions corresponding to the areas marked by boxes in photomicrograph represent labeled fibers that were found in 78 consecutive sections, covering about 2.3 mm. Also illustrated is an alternative path of fibers through an emissary canal (top left). Blue areas depict bone marrow. Red line indicates the plane of analysis. PAG, periaqueductal gray.

Figure 12.

Horizontal view of CGRP-labelled fibers in the sagittal, coronal and lambdoid sutures of the adult calvaria, just above the eye level. At this level (schematic illustration of the skull and photomicrograph), labelled fibers appear to concentrate distinctly in the sutures while absent from the diploë, as shown in the camera lucida illustrations. Reconstructions corresponding to the areas marked by boxes in photomicrograph represent labeled fibers that were found in 17 consecutive sections, covering about 0.5 mm. Note the high density of fibers in the coronal suture on both sides of the skull. Blue areas depict bone marrow. Red line indicates the plane of analysis.

Figure 13.

Horizontal view of CGRP-labelled fibers in the sagittal, coronal and lambdoid sutures of the adult calvaria at the level of the eyes. At this level (schematic illustration of the skull and photomicrograph), labelled fibers are distinctly concentrated in the endosteal side of the coronal sutures, as shown in the camera lucida illustrations. Reconstructions corresponding to the areas marked by boxes in photomicrograph represent labeled fibers that were found in 61 consecutive sections, covering about 1.8 mm. Blue areas depict bone marrow. Red line indicates the plane of analysis.

Figure 14.

Horizontal view of CGRP-labeled fibers in the squamos suture in the adult calvaria, just below the eye level. At this level (schematic illustration of the skull and photomicrograph), labelled fibers are distinctly concentrated in the endosteal side of the squamos sutures, as shown in the camera lucida illustrations. Reconstructions corresponding to the areas marked by boxes in photomicrograph represent labeled fibers that were found in 78 consecutive sections, covering about 2.3 mm. Note presence of labelled fibers in the cavities of the bone marrow. Blue areas depict bone marrow. Red line indicates the plane of analysis. 3V, third ventricle.

Figure 15.

Points of entry of nerve fibers into the sutures of the adult calvaria. Peripherin (A, B) and CGRP (C, D) nerve fibers in the proximal side of the sutures were observed to branch out of nerve bundles in the dura. CGRP nerve fibers in the distal side of the suture did not branch out of bundles in the periosteum (E, F). Arrowheads point to labelled fibers. A-D, F – horizontal plane; E – coronal plane. Scale bar: A-E – 50 µm; F – 25 µm.

Figure 16.

Extensive innervation of the periosteum by peripherin (A) and CGRP fibers (B, C). A, innervation of periosteum overlying the parietal bone at the top of the adult skull. B, innervation of periosteum overlying the interparietal bone at the back of the skull. C, innervation of periosteum overlying the frontal bone on the side of the skull. Note that periosteal fibers do not enter the calvarial bones. Arrowheads point to labelled fibers. A – coronal plane; B, C – horizontal plane. Scale bar: A – 50 μm ; B – 500 μm ; C – 200 μm .

Figure 17.

Extracranial origin of periosteal innervation in the adult calvaria. Peripherin (A, coronal plane) and CGRP fibers (B, horizontal plane) in the galea aponeurotica issuing collateral branches to the periosteum. Arrowheads point to labelled fibers. Scale bar: A – 100 µm; B – 50 µm.

Figure 18.

CGRP nerve fibers traversing the arachnoid between the dura and pia in the calvarial leptomeninges of the mouse pup. Arrowheads point to labelled fibers. Scale bar: A – 100 μ m; B, C – 50 μ m. All sections are coronal.

Figure 19.

Hypothetical implications to migraine headache. A. Extracranial origin of intracranial pain. In this scenario, action potentials generated at extracranial collaterals of meningeal pain fibers (1) spread antidromically to collaterals that terminate inside the cranium, resulting in local release of proinflammatory neuropeptides and activation of neighboring meningeal nociceptors (2). B. Intracranial origin of extracranial pain. In this scenario, action potentials generated at intracranial meningeal pain fibers (1) spread antidromically to collaterals that terminate outside the cranium (2), resulting in local release of proinflammatory neuropeptides in the scalp and activation of neighboring somatic nociceptors (3). Asterisk marks original site of activation. Red dots represent local release of inflammatory neuropeptides (e.g., CGRP, substance P), Red cylinder depicts a blood vessel (e.g., temporal artery). TG, trigeminal ganglion.