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The sensory innervation of the calvarial periosteum is nociceptive and contributes to headache-like behavior

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Abstract

Headaches are thought to result from the activation and sensitization of nociceptors that innervate deep cephalic tissues. A large body of evidence supports the view that some types of headaches originate intracranially, from activation of sensory neurons that innervate the cranial meninges. However the notion of an extracranial origin of headaches continues to be entertained, although the identity of deep extracranial cephalic tissues which might contribute to headaches remains elusive. Here we employed anatomical, electrophysiological, and behavioral approaches in rats to test the hypothesis that the sensory innervation of the calvarial periosteum is nociceptive. Neural tracing indicated that the calvarial periosteum overlying the frontal and parietal bones is innervated primarily by small and medium-sized neurons in the trigeminal ganglion's ophthalmic division. In vivo single unit recording in the trigeminal ganglion revealed that calvarial periosteal afferents have slowly conducting axons, are mechanosensitive and respond to inflammatory mediators, consistent with a nociceptive function. Two distinct neuronal populations were distinguished based on their peripheral axonal trajectory: one that reached the periosteum through extracranial branches of the trigeminal nerve, and another that took an intracranial trajectory, innervating the cranial dura and apparently reaching the periosteum via the calvarial sutures. In behavioral studies, inflammatory stimulation of these afferents promoted periorbital tactile hypersensitivity, a sensory change linked to primary headaches. Activation and sensitization of calvarial periosteal afferents could play a role in mediating primary headaches of extracranial and perhaps also intracranial origin, as well as secondary headaches such as post-craniotomy and post-traumatic headaches. Targeting calvarial periosteal afferents may be effective in ameliorating these headaches.

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1. Introduction

Headaches are believed to occur as a result of the activation and increased sensitivity of primary afferent nociceptive neurons that innervate deep cephalic tissues. Based on a large body of work, the trigeminal sensory innervation of the cranial meninges and their related large blood vessels has been implicated as the major sensory system that mediates primary and secondary headaches of intracranial origin [25,31]. Nonetheless, the notion that there might be an extracranial cephalic origin for the headache, as proposed more than 70 years ago [27], continues to be entertained [25] and is supported in part by the findings that some types of headaches can be diminished or prevented by treatments that interfere with neural traffic in peripheral afferent nerves innervating extracranial cephalic structures [1,18].

The notion that some headaches have an extracranial origin raises the critical question, which deep cephalic tissues might contribute to the pain? Pericranial muscles were hypothesized to play a role in headache, in part due to the finding of muscle tenderness during an attack [14], but whether nociceptive activation of pericranial muscle afferents innervation can lead to a headache remains uncertain [13]. Extracranial cephalic arteries, such as the superficial temporal artery, have also been suggested to be a source of headache [25]. However, there is currently little direct evidence to support such a role.

Although the intracranial and extracranial sensory innervations of the head have been considered as separate entities [24], two recent anatomical studies have suggested that the calvarial periosteum, an extracranial deep scalp tissue, which presumably receives most of its sensory innervation from nerves that innervate the scalp [16], is also innervated by afferent axons that take an intracranial trajectory which passes through the cranial dura and then penetrates the calvarium, possibly through the calvarial sutures [17,29]. It has been suggested that these afferents have a dual innervation territory, in that they apparently supply both the periosteum and the cranial dura [29]. It thus appears that the intracranial and extracranial sensory innervations are not entirely separate, and therefore there may be shared intra- and extracranial mechanisms of headache. While the sensory innervation of the calvarial periosteum could potentially contribute to headache, whether of intracranial or extracranial origin, our present knowledge of this innervation is confined to anatomical studies, and there is presently no physiological or behavioral evidence relating to the possible nociceptive properties of this innervation and its potential contribution to headache. Therefore, the present study was undertaken in order to resolve the following unanswered questions: what are the sensory properties of afferents that innervate the calvarial periosteum? Does the extracranial sensory innervation of the periosteum differ from the one that presumably arises intracranially? Does the activation of this putative nociceptive innervation of the periosteum lead to the development of periorbital tactile hypersensitivity, which can be linked to headache [2,7]?

2. Materials and methods

2.1 Animals

Sprague-Dawley male rats (250-350 g) were used in compliance with the experimental protocol approved by the institutional Animal Care and Use Committee of the Beth Israel

Deaconess Medical Center and Harvard Medical School and adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain [34].

2.2 In vivo retrograde neural tracing of trigeminal afferents that innervate the calvarial periosteum

Animals were anesthetized with a mixture of ketamine/xylazine. Under aseptic conditions, the skin and connective tissue overlying the calvarial bone were cut longitudinally along the midline through the aponeurosis (Galea aponeurotica) and 5 μ l of the retrograde tracer fluorogold (FG, 1% in ddH₂O, Fluorochrome) were injected into the periosteum either ~4 mm rostral or ~5 mm caudal to the bregma suture line using a Hamilton syringe with a 25 gauge needle. To avoid the spreading of the tracer to the overlying scalp tissue, a piece of parafilm was glued above the injected site using cyanoacrylate and the skin was sutured. Animals were allowed to recover for 4 days and then were deeply anesthetized and perfused transcardially with 0.1M phosphate buffer saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1M PBS. The trigeminal ganglion (TG) ipsilateral to the injection site was dissected, cryoprotected in PBS containing 30% sucrose at 4°C for 24 hours, embedded in optimum tissue cutting medium (OCT, Tissue tek) and sectioned at 25 μ m using a cryostat. Sections were mounted on glass slides and FG-labeled cells were visualized using excitation/emission filters at 360/408 nm (blue) and captured at 20 $\times$. Cross-sectional somatic area was measured using NIH ImageJ software. To avoid counting the same cells on successive sections, only labeled cells in which the nucleus was fully visible were counted.

To visualize the periosteal afferents in the vicinity of the FG injection site, we lifted a full thickness scalp flap above the frontal and parietal bones of the perfused animals using a 2 mm dental periosteal elevator. The flap was removed and immersed for post-fixation in PBS containing 4% paraformaldehyde at 4°C for 24 hours. Fixed flaps were then cryoprotected with 30% sucrose, frozen in OCT and 20 micron cryostat sections were then made through the periosteum, galea and skin and thawed onto slides. The sections were permeabilized with PBS containing 0.1% Triton X-100 and then subjected to fluorescent immunohistochemistry to label thinly myelinated and unmyelinated primary afferent neurons using a polyclonal rabbit anti-peripherin antibody (Chemicon, Temecula, CA, AB1530 diluted 1:5,000) [11]. After washing with PBS, the sections were incubated with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Grand Island, NY, A-11008, 1:2000) and Cy3-conjugated avidin (Rockland, Gilbertsville, PA, A-003-04, 1:1000) to visualize periosteal mast cells.

2.3 Electrophysiological recordings

Rats were deeply anesthetized with Urethane (1.5 g/kg i.p.) and then placed in a stereotaxic apparatus. Extracellular recordings of primary afferent units with calvarial periosteal receptive fields (RF) were made from their cell bodies in the TG using a platinum-coated tungsten microelectrode that was inserted into the ganglion through a craniotomy made in the contralateral calvarium using an angled (22°) approach. To search for trigeminal afferents with calvarial periosteal RF, we used an electrical search stimuli (0.5 ms pulse, 0.5-5 mA, 0.5 Hz) with the stimulating electrode moved throughout the exposed periosteum. During the experiments, action potentials were acquired using a real-time waveform

discriminator (Spike 2, CED, Cambridge, UK). To prevent drying, the exposed tissue was kept moist with modified synthetic interstitial fluid (SIF; 135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 5 mM CaCl₂, 10 mM glucose and 10 mM HEPES, pH 7.2).

2.4 Assessments of ongoing activity, mechanosensitivity and responsiveness to chemical nociceptive stimuli

Following the identification of an afferent, its baseline ongoing activity was recorded for 30 min. A neuron was considered as having a basal level of ongoing activity if it fired at least 6 action potentials (1 spike/5 min) during the baseline period. To assess mechanosensitivity, a custom-made glass probe was used as a search stimulus. Upon identification of a mechanosensitive periosteal RF, von Frey monofilaments were employed to determine the most sensitive spot and semi-quantitatively evaluate its mechanical activation thresholds. Quantitative assessments of the afferents' mechanical responsiveness were then examined using a feedback-controlled mechanical stimulator (Series 300B, Aurora Scientific, Aurora, ON) fitted with a flat-ended plastic cylinder (0.5 mm) positioned ~1 mm above the most sensitive spot. Stimulus trials for testing mechanosensitivity consisted of graded square-wave stimuli (100-msec rise time, 2-sec width, 60-sec inter-stimulus interval) delivered in ascending order. Each trial included one threshold stimulus (which normally gave rise to 1-2 action potentials) followed by 2 suprathreshold stimuli (usually X2 and X4 of the threshold). Throughout the study, mechanical stimuli trials were applied every 15 min. Based on its response to a suprathreshold mechanical stimuli, we defined an afferent as rapidly adapting if a response (normally <2 spikes) was elicited only during the initial presentation and/or removal of the stimulus. An afferent was considered as slowly adapting, if firing was maintained for at least 1 sec during the 2 sec stimulus (50% of the stimulus duration).

To determine the chemosensitivity of periosteal afferents, following a 30 min baseline period, we recorded changes in ongoing activity in response to 10 min application of SIF containing potassium chloride (50-500 mM), low pH SIF (pH 6 or 5), or capsaicin (10 µl). Agents were applied topically to the exposed periosteum in a volume of 50 µl. Each afferent was exposed to no more than 2 different chemical stimuli (in a random order) with the second stimulus applied at least 2 hours after the first one. In the case of KCl and low pH, doses were presented in ascending order with intervals of at least 1 hour between the doses. Because many afferents demonstrated poor responsiveness to capsaicin and low pH, to discount the possibility that such lack of responses was due to poor diffusion, we retested the affects of capsaicin and pH 5 in non-responsive afferents, 3 hours later by recording changes in afferent activity in response to their injecting (5 µl, same concentration as used for the topical application) into the periosteum ~0.5 mm from the RF using a Hamilton syringe fitted with a 27 gauge needle.

To test whether afferents with periosteal RF can become activated or mechanically sensitized following their exposure to inflammatory mediators (IM), we examined changes in ongoing activity and mechanosensitivity in response to 10 min topical application of a mixture of histamine, serotonin and bradykinin (at 100 µM) and PGE₂ (at 10 µM) made in SIF. The responses to IM were tested in neurons that were not treated previously with another nociceptive agent. In this paradigm, baseline ongoing activity and responses to

mechanical stimulation were determined during at least 3 consecutive trials prior to the treatment with the agents. Changes in ongoing activity and/or mechanosensitivity were then determined for 60 min following the application of the IM to the periosteum.

2.5 Characterization of extracranial vs. intracranial axonal trajectories of trigeminal afferents neurons that innervate the calvarial periosteum

To investigate whether trigeminal afferents that innervate a specific periosteal RF originate from extracranial nerves that innervate the scalp we locally injected 2% lidocaine (~5 μ l) near one of the 3 major nerves that innervate the V1/V2 region of the scalp and which are also likely to innervate the periosteum overlying the frontal and temporal bones, namely the supraorbital (V1) and supratrochlear nerves (V1) and the zygomaticotemporal nerve (V2). To determine whether a given neuron with a periosteal RF originated intracranially, we took the following steps: in units in which electrically or mechanically evoked responses were not blocked by the extracranial lidocaine administration we made stepwise incisions, in order to cut all possible extracranial innervation of the periosteal RF from its extracranial innervating nerve, ending up with a small (~2 mm²) square periosteal “island” with a responding RF localized in the middle. We considered these neurons to receive innervation originating intracranially, likely in the meninges. To further explore the origin of these neurons’ sensory innervation, we removed the periosteum and performed a craniotomy to explore the presence of dural RF using mechanical and electrical stimulation of dura.

2.6 Behavioral sensory testing

The development of periorbital hypersensitivity was tested using a method described initially by Oshinsky and Gommonchareonsiri [26]. Briefly, the animals were placed in a transparent flat-bottomed acrylic holding apparatus (85 mm diameter $\times$ 204 mm length) that was large enough for the animals to escape the stimulus. Prior to testing, the animals were allowed to acclimatize to the testing apparatus for at least 30 min. To examine whether animals developed static (punctuate) pericranial mechanical pain hypersensitivity, the skin region, which included the midline area just above the eyes and about 1 cm posterior, was stimulated with different von Frey (VF) filaments (18011 Semmes-Weinstein Anesthesiometer Kit) that exert forces between 1.2-15 grams. Behavioral testing was conducted every 1 hour, at baseline, and then at 1, 2, 3, and 4 hours after the administration of 5 μ l of the IM mixture used in the electrophysiological studies above, using a Hamilton syringe with a 27 gauge needle, about 3 mm lateral and 3-4 mm caudal to bregma. In vehicle control studies, SIF was injected in a similar fashion. Changes in tactile skin sensitivity were evaluated as in our previous work [20] by recording four behavioral elements adapted from the work of Vos et al. [32] as follows: (1) *Detection*: the rat turned its head towards the stimulating object and the latter was explored (usually by sniffing); (2) *Withdrawal*: the rat turned its head away or pulled it briskly from the stimulating object, had a brisk hind paw withdrawal or moved away from the stimulating object; (3) *Escape/attack*: the rat turned its body briskly in the restrainer or attacked (biting and grabbing movements) the stimulating object; (4) *Face/body grooming*: the rat displayed at least 3 uninterrupted series of grooming strokes directed to the stimulated area. Starting with the lowest forces, each VF hair was applied 3 times (intra-trial interval 10 sec) and the behavior that was elicited at least twice was recorded. For statistical analysis, the score recorded was based on the most aversive

behavior noted. The force that elicited a withdrawal response was considered as the mechanical pain threshold. To evaluate pain behavior in addition to changes in threshold, for each rat, at each time point, a cumulative response score was determined by combining the individual scores (0-4, table 1) for each one of the VF filaments tested. All tests were conducted and evaluated in a blinded manner.

2.7 Statistical analyses

Statistical analyses were conducted using Statview (SAS institute). Data is presented as either mean $\pm$ SE or as box plots (median $\pm$ 5th, 25th, 75th, and 95th percentiles). Differences in response properties between units with different conduction velocities (CV) and trajectories were analyzed using Mann Whitney test or Fisher's exact test. The development of inflammatory-related changes in the response of periosteal afferents was analyzed using the Wilcoxon Signed Rank test. For the behavioral studies, changes in VF thresholds and cumulative pain scores were analyzed initially using the Friedman test. When this test was significant, the Wilcoxon Signed rank test was applied to determine the increase in pain behavior onset latency and duration. The level of significance was set at $p=0.05$.

3. Results

3.1 Localization and size distribution of TG cell bodies that terminate in the calvarial periosteum overlying the frontal and dorsal temporal bones

We used local periosteal application of the retrograde tracer FG to study the localization and size distribution of trigeminal afferents innervating the calvarial periosteum rostral to the lambdoid suture. Importantly, FG injections were made in an area of the periosteum, in which the overlying musculo-fibrous aponeurosis layer does not receive sensory innervation (see Fig. 1), thus limiting the labeling of sensory neurons that innervate cranial muscular tissues. All FG-labeled neurons were observed ipsilateral to the injection side. Application of FG to the periosteum 5 mm rostral to the coronal suture ($n=4$) resulted in a total of 544 labeled neurons, with an average of 136 neurons per animal (range 99-177). FG applied 5 mm rostral to the lambdoid suture ($n=4$) resulted in a similar number of total labeled neurons (578, average 144.5, range, 115-167). Most of the labeled TG cells (95.6 %) were distributed within the ophthalmic (V1) region (Fig. 1C). The remainder was in the maxillary (V2) region. The median cross-sectional area of the labeled neurons was $749 \mu\text{m}^2$ (range 161-2757). As Fig. 1D demonstrates, the great majority of the labeled cells were small ($<30 \mu\text{m}$ diameter, 45.5%) or medium-size ($30\text{-}40 \mu\text{m}$ diameter, 39.8%). There were no significant differences between the populations of cells labeled from tracer injection in the rostral vs. caudal site in number or size distribution of labeled cells.

3.2 Response properties of trigeminal afferents that innervate the calvarial periosteum

3.2.1 Conduction velocities and basal ongoing activity—We recorded from a total of 115 trigeminal units that could be activated by electrical stimulation of the calvarial periosteum. Of those, we identified units with conduction velocities (CV) in the range of A β (CV > 12 m/sec, median 16 m/s, IQR 10.35, $n=7$), A δ ($1.5 < \text{CV} < 12$ m/sec, median 5.45 m/sec, IQR 3.67, $n=79$) and C afferents (CV < 1.5 m/sec, median 1.09 m/s, IQR 0.75, $n=29$, Fig 2A). Among all of the units recorded, varying rates of ongoing activity were present in

14% of the A δ units and 78% of the C units. None of the A β units displayed ongoing activity. As Fig. 2B demonstrates, when compared to the A δ population, C units had a higher propensity to display ongoing activity ($p < 0.001$). However, when including in the analysis only neurons with ongoing activity, there was no difference between the ongoing activity rate in the A δ and C- units.

3.2.2. Mechanosensitivity—Nearly all (113/115) of the units that could be activated by electrical stimulation of the periosteum had a mechanosensitive RF within the ipsilateral periosteal area extending from the lambdoid suture to about 6 mm rostral to the coronal suture. Most (93/113, 82%) of the mechanosensitive afferents were slowly-adapting while the rest were rapidly-adapting (Fig. 2D,E). Rapidly-adapting responses were displayed by 30% of the A β , 25% of the A δ , but none of the C units. As shown in Fig. 2F, the lowest mechanical thresholds were noted in the A β population (median 0.4 g, IQR 0.13), followed by the A δ (median 1 g, IQR 1.6) and C-units (median 1.4 g, IQR 1.6).

3.2.3. Axonal trajectories of trigeminal calvarial periosteal afferents and their relationship to the afferent's basic response properties—In addition to the presumed extracranial innervation of the periosteum by nerves that innervate the scalp [16], two recent studies in rodents suggested that this calvarial lining tissue also receives sensory innervation from collaterals of afferents that innervate the dura mater [17,29]. To further examine the extracranial vs. intracranial origin of the periosteal innervation, we investigated the axonal trajectories of the periosteal sensory innervation in 86 trigeminal afferent units using selective local anesthetic blockade of individual extracranial nerves. As predicted, two major axonal trajectories were identified, extracranial and intracranial. Most (60/86, 70%) of the afferents with periosteal RFs had extracranial axonal trajectories with the supraorbital ($n=46$, 53%), the supratrochlear ($n=7$, 8%) and the zygomaticotemporal ($n=7$, 8%) nerves identified as the supplying extracranial nerves. In the remaining 26 (30%) units, the response to periosteal stimulation was not affected by the peripheral nerve blockade, and an additional RF could be identified on the dura, indicating that the afferent reached the periosteum via an intracranial axonal trajectory. While in units with extracranial axonal trajectories the RFs were distributed throughout the periosteum, in most (21/26) of the units with presumed intracranial axonal trajectories, the RFs were localized above or in very close proximity (< 1 mm) to a suture line (Fig. 3A). In these suture-related afferents, a dural RF could also be identified, primarily on the venous sinus most closely related to the suture line. The remaining 5 afferents with presumed intracranial axonal trajectories had periosteal RFs very near or just above an emissary canal. These neurons also had dural RFs just below the emissary canal. Afferents with extracranial axonal trajectories had faster CVs than those with intracranial trajectories (median 4.1 ms/IQR 5.6 extracranial vs 3.1 ms/IQR 4.7 intracranial, $p < 0.05$), likely due to the presence of more afferents with A β CVs (6 extracranial vs. 1 intracranial, $p < 0.05$). When A β units were omitted from the analysis, there was no statistical difference in the CVs between the extracranial and intracranial projecting afferents. When comparing only afferents with CVs at the nociceptive range (slowly conducting A δ and C), those with extracranial trajectories had lower mechanical thresholds (median 0.8 g, IQR 1.6 extracranial vs. 1.4 g, IQR 4.1 intracranial; $p < 0.01$, Fig. 3C). All mechanosensitive units with an intracranial axonal trajectory as well as most (89%) of the

units that solely innervated the calvarial periosteum (i.e. had extracranial axonal trajectories) were slowly adapting. The percentage of units with ongoing activity as well as its rate nonetheless was no different between units with extracranial vs. intracranial trajectories.

3.2.4. Chemosensitivity and inflammatory sensitization of periosteal afferents

—To examine a potential role for periosteal afferents as chemo- and inflammatory sensing nociceptors, we tested the effect of application of algescic or inflammatory agents to their periosteal RF. Application of the algescic substance potassium chloride (KCl) activated periosteal afferents dose-dependently: 11/37 (29.7%), 17/32 (53.1%), and 25/31 (80.6%), were activated by KCl at 50, 200, and 500mM, respectively. There was no difference in the percentage of KCl-responsive units between A δ vs. C-units, or between units with extracranial vs. intracranial axonal trajectories.

To examine the responses of periosteal afferent units to low pH, we tested changes in the activity of 20 units (11 A δ , 9 C) following local application of SIF with a pH 6, which has been shown to activate dural afferents in vitro [33]. In response to this acidic stimulus only 2 A δ and 1 C unit were activated, and this activation was of rather low magnitude (<10 spikes/5 min). Further testing of a more acidic solution (pH 5) revealed similar weak responses in only 3/16 A δ and 0/9 C units, all of which had extracranial trajectories.

Capsaicin sensitivity was tested in 12 A δ and 14 C units, using periosteal administration of a 10 μ l, a dose which has been shown to be effective in activating nociceptive afferents that innervate the skin [8] as well as deep tissues, including the colon [6] and dura mater [5,10]. Units with calvarial periosteal RFs demonstrated very poor responsiveness to capsaicin with only 1/12 A δ (with an extracranial axonal trajectory) and 3/14 C-units (2 with extracranial axonal trajectories) demonstrating a brief (< 2 min) activation. In 2 non-responsive units with intracranial trajectories, application of capsaicin to their exposed dural RF, 2 hours following the periosteal application promoted neural activation, suggesting that the intracranial and extracranial branches of these afferents may have different chemosensitive properties.

Responsiveness to inflammatory mediators is a hallmark of nociceptive afferents. We therefore tested whether local application of a mixture of the nociceptive inflammatory mediators (IM) histamine, serotonin and bradykinin (at 100 μ M) and PGE2 (at 10 μ M), which we have shown earlier can promote activation and sensitization of dural nociceptors [21] could also lead to the activation and/or increase in the mechanical responsiveness of trigeminal afferents with periosteal RFs. Overall, activation was noted in 31% (12/38) of the A δ and 47% (8/17) of the C units. A higher percentage of units with an extracranial axonal trajectory (19/42) became activated by IM when compared to units with intracranial trajectories (1/12, $p < 0.001$). Local IM application gave rise to an increase in ongoing activity that started during the time of the application and persisted for at least 30 min (Fig. 4C). IM application led to mechanical sensitization in 40% (4/10) of the A δ units and 100% (6/6) of the C units. This sensitization lasted for at least 30 min for the threshold responses and 15 min for the suprathreshold responses (Fig. 4D, E, $p < 0.05$). IM-induced mechanical sensitization was found in 70% (7/10) of units with an extracranial axonal trajectory and 33% (2/6) of units with an intracranial trajectory (N.S.).

3.4. Development of periorbital cutaneous mechanical hypersensitivity following inflammatory stimulation of calvarial periosteal afferents

Given that periosteal application of IM was able to activate and sensitize trigeminal afferents with calvarial periosteal RFs, we tested whether such responses could promote a sensory change reminiscent of headache in humans, namely the development of periorbital cutaneous mechanical hypersensitivity [2,7]. As Fig. 5 demonstrates, injection of a sensitizing dose of IM into the periosteum elicited significant decreases in periorbital cutaneous mechanical thresholds over time ($p < 0.01$). A post hoc analysis revealed significant decreases at 2 and 3 hours post-IM administration ($p < 0.05$). The change in mechanical withdrawal thresholds was accompanied by an increase in the cumulative pain responses over time ($p < 0.05$) that was also delayed and different than baseline at 2 and 3 hours post IM treatment ($p < 0.05$, Fig. 5B). In vehicle control studies, periosteal administration of SIF affected neither periorbital mechanical thresholds nor the cumulative pain responses to periorbital mechanical skin stimulation.

4. Discussion

Headaches such as migraine are thought to arise intracranially, but some clinical data has led to the speculation of an additional extracranial origin for headache. One extracranial tissue that may play a role in mediating headache is the calvarial periosteum. Here, we provide anatomical and electrophysiological evidence that the calvarial periosteum overlying the frontal and parietal bones is innervated by slowly conducting mechano- and chemosensitive trigeminal afferents with nociceptive properties. Data from our behavioral study further indicate that inflammatory-related activation and sensitization of trigeminal periosteal afferent neurons can give rise to periorbital tactile hypersensitivity, an observation reminiscent of the cephalic sensory changes seen in humans during primary headache attacks, namely periorbital tactile hypersensitivity [2,7]. Taken together, our findings support the view that the trigeminal nociceptive innervation of the calvarial periosteum could contribute to primary headaches. We further propose that activation and sensitization of calvarial periosteal nociceptors may also be involved in the pathophysiology underlying secondary headaches in cases where the periosteum and its innervation become compromised such as following a craniotomy (post-craniotomy headache) or following a closed head injury (post-traumatic headache).

Previous anatomical findings suggest that extracranial sensory nerves that innervate the scalp skin send collaterals that pierce the galea aponeurotica and thus presumably terminate in the calvarial periosteum [15,16]. Our anatomical tracing study provides new data indicating that the sensory innervation of the calvarial periosteum overlying the dorsal parts of the frontal and parietal calvarial bones is supplied primarily by small and medium diameter trigeminal neurons with cell bodies located mainly within the ophthalmic (V1) division of the trigeminal ganglion, a distribution that is consistent with the ophthalmic referral pattern of migraine and some other headaches. Data from our electrophysiological study, which employed extracranial anesthetic blockade, provides evidence that the majority of these periosteal trigeminal afferents has an extracranial ophthalmic axonal trajectory, primarily through the supraorbital nerve, a major V1 branch.

The findings that trigeminal afferents that innervate the calvarial periosteum had slow conduction velocities, slowly adapting responses to mechanical stimuli, and were activated and sensitized following administration of pro-inflammatory mediators to their RF support their involvement in nociception. That such inflammatory stimulation of periosteal afferents promoted the development of periorbital tactile hypersensitivity, a sensory change that accompanies primary headaches including migraine [2], further points to their potential involvement in headache. The development of periorbital tactile allodynia in primary headaches occurs most likely as a result of central sensitization of second-order neurons in the spinal trigeminal nucleus. That the duration of the pericranial cutaneous hypersensitivity evoked by the periosteal IM application was longer than the peripheral sensitization evoked by a similar stimulus strongly implicates central sensitization as the mechanism that mediated this change in periorbital cutaneous sensitivity.

In addition to the identification of periosteal nociceptive afferents that projected through extracranial nerves, we also encountered a smaller population of afferents (~30%) with periosteal RFs that were not blocked by extracranial local anesthesia. Our data suggest that these afferents likely reach the periosteum through an entirely intracranial axonal trajectory that passes through the dura and exits the calvarium primarily via the sutures but also through emissary canals, as suggested recently by an anatomical study [17]. Importantly, these afferents had an additional RF on the intracranial dura and their periosteal RFs were more restricted than those of neurons that lack a dural RF, in that they were present primarily along the calvarial sutures line overlying the dural venous sinuses and above emissary canals. Our findings add to those of a recent *in vitro* study [29] that studied afferents in the spinosus branch of the mandibular nerve that had RFs on the periosteum underlying the temporalis and was able to identify in 20% of these afferents another RF localized to the intracranial dura. It should be noted that the presence of TG afferents with two branches that innervate the dura and calvarial periosteum is consistent with the branching of deep paraspinal nociceptors, which innervate more than one type of non-cutaneous tissue [4].

In addition to their conceivable role in mediating headache, it is possible that intracranial trigeminal afferents that cross the calvaria through the sutures and innervate the periosteum are also important to the process of cranial suture closure during the early stages of cranial development [19]. In humans, this sensory innervation is likely to be pruned above suture lines that become fused later in life. The intracranially derived periosteal innervation that is most likely to remain intact during adulthood and thus, could potentially play a role in headache, is the one that reaches the periosteum through calvarial sutures with relatively higher levels of patency, in particular the lambdoid suture [28].

In addition to their conceivable role in mediating primary headaches, the innervation of the calvarial periosteum may also contribute to some secondary headaches in which this tissue is injured and becomes inflamed. The pulsating or pounding headache that occurs in about two-thirds of patients undergoing a craniotomy [9,23] could result from damage to extracranial periosteal afferents, given that this headache can be alleviated by regional scalp nerve block [12,23]. The sensory innervation of the calvarial periosteum may also contribute to the development and maintenance of other calvarial-related head pain such as following

head trauma. The persistence of post-traumatic headaches, which can last more than a year after the injury [22], may be due to processes related to acute and/or chronic damage to periosteal afferents or local inflammation, two processes which could lead to the ongoing activation and sensitization of these afferents. The supraorbital nerve, which our study shows innervates a large portion of the calvarial periosteum in the rat, and likely also in humans [16], has been proposed to play a role in post-traumatic headache [30]. It will be of interest to examine whether manipulations that lead to blockade of the sensory innervation of the calvarial periosteal innervation, whether having trigeminal or cervical origins, can be effective in reducing post-traumatic headaches.

Extracranial anesthetic blockade of neural traffic in peripheral afferent nerves that innervate extracranial structures as well as less invasive manipulations such as manual therapies thought to decrease afferent input have been suggested to provide headache relief [1,3,18]. It will be of interest to examine whether similar therapeutic approaches targeted towards the calvarial periosteal sensory innervation can be effective in reducing post-traumatic headaches.

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The calvarial periosteum is innervated by nociceptors with extracranial and intracranial axonal trajectories. Activation of these afferents could contribute to primary and secondary headache such as post-traumatic headaches.

Fig. 1.

Localization and size distribution of calvarial periosteal projecting primary afferents in the TG. (A) A cross section through the scalp at a level between Bregma and Lambda (the area where the retrograde tracer FG was injected) showing peripherin-IR nerve fibers in the periosteum and skin. Note the lack of galea innervation suggesting that afferents that innervate the periosteum do not send collaterals that innervate more dorsal scalp tissues. (B) A high magnification image of a cross section through the periosteum showing peripherin-IR afferent nerve bundles (arrows) as well as periosteal mast cells (MCs, arrowheads). (C) Drawings of horizontal TG sections showing the distribution of retrograde-labeled neurons that project to the calvarial periosteum. Note the predominantly ophthalmic (V1) distribution. (D) Size distribution of TG cells that project to the calvarial periosteum. Data is the mean $\pm$ SEM of the cross-sectional areas of the labels cells obtained from 8 experiments. (E) Based on their cell diameters, the vast majority of the calvarial periosteal projecting cells were small or medium size.

Fig. 2.

General characteristics of trigeminal afferents that innervate the calvarial periosteum. (A) Box plots of the CVs of trigeminal afferents that innervate the calvarial periosteum. Note that most units had CVs within the range of small diameter Aδ and C peripheral sensory neurons (CV < 12 m/sec). (B) All units with ongoing activity belonged to the Aδ or C unit category. On average there were more C-units with ongoing activity. However, when comparing only units with ongoing activity there was no difference in the rate between the Aδ and the C units (C). In response to a 2 sec mechanical stimulus (upper traces in D and E) most afferents demonstrated a slowly adapting responses pattern (D) with the rest showing a rapidly adapting firing pattern (E). (F) The Aβ afferents had the lowest mechanical thresholds (* $p < 0.05$, ** $p < 0.01$ Mann Whitney test, ## $p < 0.01$ Fisher's exact test).

Fig. 3.

(A) A map of the RFs of a representative sample of periosteal innervating afferents recorded in the left TG and their central axonal trajectories, as determined by local anesthetic block of individual nerves. Most of the RFs belonged to afferents that projected centrally through the supraorbital nerve. A number of neurons with periosteal RFs did not project through an extracranial nerve and had an additional RF in the dura mater indicating an intracranial axonal trajectory. Most of these units had RFs located above the calvarial suture lines. A very few of the intracranially projecting afferents had RFs above an emissary canal. Overall CVs (B) and the level of ongoing activity (C) did not differ between extracranially and intracranially projecting units. (D) Extracranially-projecting periosteal afferents however had lower mechanical thresholds than intracranially projecting units. (* $p < 0.05$ Mann Whitney test).

Fig. 4.

TG afferents that innervate the calvarial periosteum become activated and sensitized in response to an inflammatory stimulus. Sample recordings of slow conducting TG afferents with periosteal RFs showing increased ongoing firing rate (A) and responses to mechanical stimulation of their periosteal RF (B) following local administration of IM. Neural responses in spikes/sec are indicated in parentheses. IM-evoked increases in ongoing activity and responsiveness to threshold mechanical stimuli persisted for at least 30 min (C, D) while the increased responsiveness to suprathreshold mechanical stimuli was shorter lasting (E). TG afferents with periosteal RFs that had extracranial axonal trajectories show a higher propensity to become activated following exposure to IM (F; * $p < 0.05$ Wilcoxon Signed

Rank test, ##, $p < 0.01$ Fisher exact test), but there was no difference in the propensity to become sensitized (G).

Fig. 5.

Development of periorbital cutaneous tactile hypersensitivity following administration of IM into the calvarial periosteum. Note the delayed development of the hypersensitivity, which was manifested as a reduction in thresholds (A) and an increase in the total behavior score (B). * $p < 0.05$, Wilcoxon Signed rank test.

Table 1
Behavioral response scoring system

Response category	<u>Observed response elements</u>				Score
	Detection	Withdrawal	Escape/attack	Grooming	
No response	–	–	–	–	0
Nonaversive response	1	–	–	–	1
Mild aversive response	–	1	–	–	2
Strong aversive response	–	–	1	–	3
Prolonged aversive response	–	–	–	1	4