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Dural fibroblasts play a potential role in headache pathophysiology

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Abstract

Nociceptive signaling from the meninges is proposed to contribute to many forms of headache. However, the events within the meninges that drive afferent activity are not clear. Meningeal fibroblasts are traditionally thought to produce extracellular proteins that constitute the meninges but do not contribute to headache. The purpose of these studies was to determine whether dural fibroblasts release factors that activate/sensitize dural afferents and produce headache-like behavior in rats. Dura mater was removed from male rats and dural fibroblasts were cultured. Fibroblast cultures were stimulated with vehicle or lipopolysaccharide (LPS), washed, and conditioned media was collected. Fibroblast media conditioned with vehicle or LPS was applied to retrogradely-labeled rat dural trigeminal ganglion neurons in vitro. Patch-clamp electrophysiology was performed to determine whether conditioned media activated/sensitized dural afferents. A preclinical behavioral model was used where conditioned media was applied directly to the rat dura to determine the presence of cutaneous facial and hindpaw allodynia. Conditioned media was also tested for interleukin-6 (IL-6) content using an ELISA. Application of LPS-conditioned fibroblast media to dural afferents produced a significant increase in action potential firing as well as cutaneous facial and hindpaw allodynia when this media was applied to the dura. Finally, stimulation of cultured fibroblasts with LPS increased IL-6 levels in the media. These findings demonstrate that fibroblasts stimulated with LPS release factors capable of activating/sensitizing dural afferents. Further, they suggest that fibroblasts play a potential role in the pathophysiology of headache.

Keywords

pain; dura; dural afferent; headache; dural fibroblasts; LPS

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Conflict of interest The authors declare that they have no conflicts of interest.

Introduction

The pathophysiology contributing to many forms of headache is not clear. Within the skull, the only pain-sensitive structure is the meninges and the only sensation induced from the meninges is pain [29]. Understanding the mechanisms leading to activation/sensitization of meningeal afferents may provide important clues into the pathophysiology of headache.

Many inflammatory mediators including acidic pH, histamine, bradykinin, prostaglandins, nitric oxide, and serotonin can activate and/or sensitize dural afferents [2; 7; 14; 23; 30; 34; 38] and the presence of these mediators in the meninges may cause headache. Mast cells and macrophages within the meninges have also been proposed to contribute to headache [11; 26; 27; 41] and these cells are a potential source of a variety of inflammatory mediators. However, the primary resident cell type in the meninges is fibroblasts. Dural fibroblasts are elongated cells with extended cell processes that show a fusiform or spindle-like shape and are oriented parallel to the flat axes of the dura mater [13]. These cells are responsible for producing the collagen, fibronectin, and other extracellular matrix proteins that make up the meninges, particularly the dura [32]. In addition to producing the dura, fibroblasts may also play a role in activating/sensitizing dural afferents via the release of pro-inflammatory substances. However, these cells have not been studied for potential contributions to headache. The purpose of these studies was to investigate whether dural fibroblasts play a potential role in the pathophysiology of headache by examining their release of factors that can activate/sensitize dural afferents.

2. Materials and methods

2.1 Animals

Male Sprague-Dawley rats (35-100g for dural fibroblast culture, 150-175g for patch clamp studies, 250-300g for behavioral studies) were maintained in a climate-controlled room on a 12h light/dark cycle with food and water ad libitum. All procedures were performed in accordance with the policies and recommendations of the International Association for the study of Pain, the National Institutes of Health guidelines for handling and use of laboratory animals, and were approved by the Institutional Animal Care and Use Committee of the University of Arizona.

2.2 Surgery

2.2.1. Retrograde tracer injection—Dural afferents were identified as previously described [39]. Briefly, seven days prior to sacrifice, animals were anesthetized with a combination of ketamine and xylazine (80 mg/kg and 12 mg/kg; Sigma-Aldrich), and two holes (3mm in diameter) were made in the skull. 5µl of Fluorogold (FluoroChrome; 4% in SIF: synthetic-interstitial fluid) was then applied onto the dura. A small piece of gelfoam was retained in the hole to increase absorption of dye and prevent dye spread out of the holes. The holes were covered with bone wax to prevent tracer leakage. Immediately postoperatively, animals received a single subcutaneous injection of gentamicin (8 mg/kg) to minimize infection. Dura at the injection sites was evaluated at the time the animals were

sacrificed and only data from animals with intact dura and no signs of damage were used for further analysis.

2.2.2. Dura cannulation—Dura cannulae were implanted as previously described. [39]. Animals were anesthetized with a combination of ketamine and xylazine (80 mg/kg and 12 mg/kg; Sigma–Aldrich). A 2 cm incision was made to expose the skull. A 1 mm hole (above the transverse sinus; 2 mm left of sagittal suture and 2 mm anterior to lambdoid suture) was made with a hand drill (Plastics One) to carefully expose the dura. A guide cannula (Plastics One), designed to extend 0.5 mm from the pedestal to avoid irritation of the dural tissue, was inserted into the hole and sealed into place with glue. Two additional 1 mm holes were made rostrally to the cannula to receive stainless-steel screws (Small Parts), and dental acrylic was used to fix the cannula to the screws. A dummy cannula (Plastics One) was inserted to ensure patency of the guide cannula. Immediately postoperatively, animals received a single subcutaneous injection of gentamicin (8 mg/kg) to minimize infection. Rats were housed separately after surgery and allowed 6–8 days of recovery.

2.3. Cell culture

2.3.1. Trigeminal ganglion culture for electrophysiology—Seven days following fluorogold application, trigeminal ganglia (TG) were removed, enzymatically treated, and mechanically dissociated as previously described [36; 39]. Rats were anesthetized with isoflurane (Phoenix Pharmaceuticals) and sacrificed by decapitation. The TG were removed and placed in ice-cold Hanks balanced-salt solution (HBSS, divalent free). Ganglia were cut into small pieces and incubated for 25 min in 20 U/ml Papain (Worthington) followed by 25 min in 3 mg/ml Collagenase Type II (Worthington). Ganglia were then triturated through fire-polished Pasteur pipettes and plated on poly-D-lysine (Becton Dickinson) and laminin (Sigma)-coated plates. After several hours at room temperature to allow adhesion, cells were cultured in a room-temperature, humidified chamber in Liebovitz L-15 medium supplemented with 10% FBS, 10 mM glucose, 10 mM HEPES and 50 U/ml penicillin/streptomycin. Cells were used within 24 h post plating.

2.3.2. Dural fibroblast culture—Rats were anesthetized with isoflurane and sacrificed by decapitation. The dura mater from 6 animals were removed and placed in ice-cold HBSS. Dura mater were cut into small pieces and incubated in collagenase A (1 mg/ml, Roche) and collagenase D (1 mg/ml, Roche) with papain (30 U/ml, Roche) for 40 to 50min. To eliminate debris, 70 µm cell strainers (BD Biosciences) were used. The dissociated cells were resuspended in DMEM/F12 (Invitrogen) containing 1× pen-strep (Invitrogen), 1× GlutaMax and 10% fetal bovine serum (Hyclone). The cells were plated in one 6-well plate (BD Falcon) and incubated at 37°C in a humidified 95% air/5% CO₂ incubator. Cultures were maintained in media until time of treatment. Dural fibroblasts were plated at a density that would achieve confluency by day 3 post-plating and cultures were used at this time point. Cultures were not passaged in any of these experiments.

2.4. Immunocytochemistry

Dural fibroblasts were cultured as described above and placed on Poly-D-Lysine coated coverslips (BD Biosciences) until 3 days post-plating. Three different cultures were

generated from 3 different sets of rats. Cells were washed with PBS, permeabilized with PBS + 10% normal goat serum (NGS) + 0.1% Triton X 100, and blocked with PBS + 10% NGS + 0.01% Na-azide. A primary rabbit glial fibrillary acidic protein (GFAP) antibody (1:1000, Cell Signaling) or a primary rabbit von-Willebrand factor (vWF) antibody (1:200, Abcam) was applied to dural fibroblast cultures for 36 hours. A primary Vimentin antibody (1:500, Millipore) or primary CD68 antibody (1:200, AbD Serotec) was applied to dural fibroblast and incubated overnight. A secondary goat anti rabbit 488 or goat anti rabbit 555 antibody (1:2000, Invitrogen) was applied for 1 hour to each group and mounted in Prolong Gold with DAPI (Invitrogen) and coverslipped. Slides were visualized using a Zeiss confocal microscope. Dural fibroblast culture images shown in Figure 2 were taken at 40X and tiled (using Zeiss ZEN) to increase the field of view. Images were histogram stretched with no gamma change. For cell counts (see Results), 3 slides were used for each antibody. From each slide, 5 random fields were chosen and images were captured at 20X. Images were histogram stretched until background staining of fibroblasts was just barely visible, and any staining around the DAPI nuclei that was above background was defined as positive staining. Staining that was not near a DAPI-labeled nucleus was not counted as positive. Total cell counts were performed using the automated DAPI count feature in ImageJ (NIH). Cell counting was performed blinded to the antibody conditions.

2.5. Fibroblast-conditioned media collection

Confluent cultures of dural fibroblasts (3 days post-plating) were treated with 5ug/ml of LPS from E. Coli (Sigma L2630) in media (DMEM/F12 with 1X GlutaMax) or vehicle (plain media) for 1 hour. After treatment, cells were washed 3 times with media to remove the LPS or vehicle. Next, 600 µl of media was added to the dishes to collect factors released from fibroblasts. The media was kept in the culture dishes for 5 hours and then collected. As a control, culture dishes with no fibroblasts were also treated with LPS and the same protocol was followed. This control determined whether any effects of the conditioned media were due to residual LPS in the media that was not removed by washes. Conditioned media was used fresh for electrophysiology experiments and snap frozen in liquid nitrogen for later use in ELISA and behavioral testing.

2.6 Electrophysiology

Whole cell patch-clamp experiments were performed on isolated rat TG using a MultiClamp 700B (Axon Instruments) patch-clamp amplifier and pClamp 10 acquisition software (Axon Instruments). Recordings were sampled at 5 kHz and filtered at 1 kHz (Digidata 1322A, Axon Instruments). Pipettes (OD: 1.5 mm, ID: 0.86 mm, Sutter Instruments) were pulled using a P-97 puller (Sutter Instruments) and heat polished to 2.5 – 4 MΩ resistance using a microforge (MF-83, Narishige). Series resistance was typically < 7 MΩ and was compensated 60-80%. All recordings were performed at room temperature. A Nikon TE2000-S Microscope equipped with a mercury arc lamp (X-Cite® 120) was used to identify FG-labeled dural afferents. Data were analyzed using Clampfit 10 (Molecular Devices) and Origin 8 (OriginLab). The pipette solution contained (in mM) 140 KCl, 11 EGTA, 2 MgCl₂, 10 NaCl, 10 HEPES, 2 MgATP, and 0.3 Na₂GTP, 1CaCl₂ pH 7.3 (adjusted with N-methyl glucamine), and was ~ 315 mosM. External solution contained (in mM) 135 NaCl, 2 CaCl₂, 1 MgCl₂, 5 KCl, 10 Glucose, 10 HEPES, pH 7.4 (adjusted with N-

methyl glucamine), and was ~ 300 mosM. Freshly thawed supernatant (600µl) was put on the TG culture for 15min and then replaced with bath solution before recording. Fluorogold positive neurons were identified and patched immediately after replacement of supernatant with bath.

2.7 Behavioral testing

Behavioral testing methods were as described previously [36; 39]. Rats were acclimated to suspended Plexiglas chambers (30 cm long × 15 cm wide × 20 cm high) with a wire mesh bottom (1 cm²). Ten microliters of vehicle or testing solution was injected through an injection cannula (Plastics One) cut to fit the guide cannula. Withdrawal thresholds to probing of the face and hind-paws were determined at 1-h intervals after administration. A behavioral response to calibrated von Frey filaments applied to the midline of the forehead, at the level of the eyes, was indicated by a sharp withdrawal of the head. Paw withdrawal (PW) thresholds were determined by applying von Frey filaments to the plantar aspect of the hind-paws, and a response was indicated by a withdrawal of the paw. The withdrawal thresholds were determined by the Dixon up-down method. Maximum filament strengths were 8 and 15 gm for the face and hind-paws, respectively. Behavioral testing was performed by an investigator blinded to the treatment conditions and unblinding took place after data analysis.

2.8 ELISA

Confluent dural fibroblast cultures were stimulated with LPS (5µg/ml) for 1 hour and conditioned media collected as described above with the following exceptions for time course studies. First, the initial 600 µl media containing LPS was collected as a 1 hour time point, cells are then washed 3 times with media, and 600 µl media was again added to the dishes for 5 hours and collected (this is the 5 hour time point, and is the same collection time as conditioned media used in electrophysiological and behavioral experiments). A rat IL-6 DuoSet ELISA Development SYSTEM (R & D Systems) was used for detecting the IL-6 level in the conditioned media. Assay procedures were performed according to manufacturer's instructions provided. Briefly, 96 well plates (Nunc) were coated with 100µl of capture antibody at 4 °C overnight and washed 3 times with wash buffer (0.05% Tween 20 in PBS) the next day. Plates were blocked with 1% BSA for 2 hours at room temperature and again washed 3 times with wash buffer. 100µL of standards or samples were immediately added to the wells and incubated at 4 °C overnight. Frozen conditioned media collected from dural fibroblasts cultures was thawed and centrifuged at 4 degrees for 15 min at 16,000 x g to remove any debris or dead cells and transferred to a fresh tube and used as samples. On the third day, samples and standards were removed and plates were washed 3 times with wash buffer. 100µl of the detection antibody was added to the wells and incubated at 4 °C overnight. On the fourth day, plates were washed 3 times with wash buffer and Streptavidin-HRP was added to the wells and incubated for 20 min at room temperature followed by 5 washes with wash buffer. The substrate solution was added and incubated for 10 min. 50 µl of stop solution (1N HCl) was added to stop the reaction and the plates were read at 450 nm with correction of 570 nm.

2.9 Data analysis

All data are presented as means $\pm$ SEM unless otherwise noted. Electrophysiology data were analyzed using linear regression for a significant difference in slopes of the lines connecting the numbers of action potentials. Behavioral studies among groups and across time were analyzed by two-factor analysis of variance (ANOVA) for treatment and time. Data were also converted to area over the time-effect curve (AOC) and analyzed with a one-factor ANOVA and Bonferroni post-test. ELISA data were analyzed by a two-factor ANOVA for treatment and time. Statistics were run using GraphPad Prism.

Results

3.1 LPS-treated dural fibroblasts release mediators that sensitize dural afferents

To explore functional interactions between dural fibroblasts and dural afferents, we examined whether primary dural fibroblasts in vitro release mediators that sensitize dural afferents. Figure 1 is a phase image of a representative dural fibroblast culture showing the typical elongated, spindle shape that is a characteristic of fibroblasts. We characterized representative samples of primary dural fibroblast cultures to ensure that these cultures were indeed fibroblasts and to examine the possible presence of other cell types. As a positive marker of fibroblasts, staining with an antibody against the intermediate filament protein vimentin was performed (Figure 2), as this protein is expressed by fibroblasts and is a common marker of this cell type. Figure 2A shows staining of the majority of the cells in the image with the vimentin antibody and cell counts found that 98.3% of the cells (5,281/5,374 cells) were vimentin immuno-positive. Staining was also performed to determine the presence of glial cells (Schwann cells), macrophages, or endothelial cells using antibodies for GFAP, CD68, or von Willebrand Factor (vWF), respectively. Staining for GFAP was found in 0.01% of cells (145/10,658), for CD68 in 0.01% of cells (76/5,854), and for vWF in 0.02% of cells (73/4,488) indicating that very few of these cell types are present in the cultures. These findings are consistent with the conclusion that the majority of these cultured cells are fibroblasts.

Retrogradely-labeled trigeminal neurons in vitro (i.e. identified dural afferents) were selected for patch clamp experiments. Slow ramp current protocols to mimic slow depolarization were used to stimulate dural afferents (Figure 3). These protocols consisted of a 1 sec ramp to 0.1 nA, followed by 3 additional 1 sec ramp protocols with a Δ = 0.2 nA ending with a final ramp value of 0.7 nA (Figure 3D). If neurons did not fire action potentials in response to this protocol, they were excluded from analysis. Dural afferents were treated for 15 min before the beginning of recording with several types of conditioned media. Fibroblast cultures were stimulated with LPS (5 μ g/mL in media) for 1 hour, LPS was removed by washing the cultures with fresh media (containing no LPS), new media (without LPS) was left in the dishes for 5 hours, and this conditioned media was collected. This group is referred to throughout the rest of this manuscript as L-CM. We also used the identical protocol but instead of LPS, we applied vehicle (plain media) for 1 hour, washed the cultures, and added fresh media that was collected after 5 hours. This group is referred to as V-CM. In order to determine whether there was residual LPS in our conditioned media that was not removed during the washes, we treated culture dishes that contained no cells

(i.e. no fibroblasts were cultured in these dishes) with LPS for 1 hour, washed the dishes, and applied fresh media for 5 hours at which point the media was collected. This group is referred to as NC-M as it is media from dishes with no cells. Cell sizes selected for recording were not significantly different between groups (L-CM: 44.39 ± 4.59 pF; V-CM: 44.76 ± 4.02 pF; NC-M: 47.98 ± 3.89 pF, $p > 0.05$). Dural afferents treated with L-CM showed a significant increase in the number of spikes compared to dural afferents treated with either V-CM or NC-M (Figure 3). At the highest ramp value (1 sec, 700 pA), dural afferents exposed to L-CM fired approximately 20 action potentials (Figure 3B) while those exposed to V-CM (Figure 3A) or NC-M (Figure 3C) fired only 1-3 action potentials (linear regression for significant difference in slopes of the lines for treatments, $F(2,70) = 15.50$, $p < 0.0001$). These results indicate that dural fibroblasts treated with LPS release mediators capable of sensitizing dural afferents.

3.2 LPS-treated dural fibroblasts release mediators that induce facial and hindpaw allodynia

In order to determine whether cultured dural fibroblasts release mediators that can produce headache-like responses in vivo, a preclinical in vivo migraine model [36; 39] was used to evaluate the effects of conditioned media on mechanical allodynia of the face and hindpaws (Figure 4). Application of L-CM to the dura produced significant time-dependent reductions in withdrawal thresholds to tactile stimuli applied to the face or the hind-paws (facial: $p < 0.005$ for treatment and $p < 0.05$ for time; paw: $p < 0.0001$ for treatment and time). Application of V-CM or NC-M did not produce facial or hindpaw allodynia. Raw data time courses from Figure 4A and 4B were also converted to AOC values in Figure 4C and 4D which showed significantly more allodynia for the L-CM group compared to V-CM and NC-M groups in both the face and hindpaws (facial: $p < 0.01$; paw $P < 0.005$). Thus, the mediators released from dural fibroblasts not only sensitize dural afferents in vitro but they also initiate events within the meninges that lead to the development of headache-like responses.

3.3 Dural fibroblasts release interleukin-6 (IL-6) in response to stimulation with LPS

We have shown previously that IL-6 sensitizes dural afferents in vitro and produces cutaneous allodynia when applied to the dura in vivo [40]. We next asked whether IL-6 is one potential mediator released from dural fibroblasts after stimulation with LPS. Application of LPS to dural fibroblasts produced a time-dependent increase in the presence of IL-6 in the conditioned media as measured by ELISA (Figure 5). During the 1 hr of LPS treatment, there was little to no IL-6 release in both the L-CM or V-CM conditions (< 10 pg/mL). However, when LPS was removed, dishes were washed, and fresh media added to the cultures for 5 hours, the same treatment time and protocol used for in vitro and in vivo experiments shown in Figures 1-3, there is significant time-dependent IL-6 release in the L-CM group (3738 ± 235 pg/mL IL-6) but not in the V-CM group (< 10 pg/mL; $p < 0.001$ for treatment and time). Thus, IL-6 is among the mediators released from dural fibroblasts and may contribute to the activating/sensitizing effects of conditioned media observed on dural afferents in vitro and to the cutaneous allodynia in the behavioral studies.

Discussion

The purpose of these studies was to test whether fibroblasts, the major cell type in the dura, can play an active role in headache pathophysiology. Pain signaling from the meninges has been proposed to be a key event in the pathophysiology of headache but little attention has been given to these cells that make the structural proteins that constitute the majority of the dura. Using several preclinical in vitro and in vivo models of headache, we report here that cultured dural fibroblasts stimulated with LPS release mediators capable of activating/sensitizing trigeminal ganglion neurons, including identified dural afferents. These data suggest that there may be conditions in which dural fibroblasts contribute to the onset of afferent nociceptive signaling from the meninges and ultimately to headache.

One of the main findings in this study is that dural fibroblasts are capable of releasing IL-6, consistent with prior studies in cardiac fibroblasts [4; 25]. IL-6 has been shown to increase in migraine patients during attacks [10; 28] and this pro-inflammatory cytokine may contribute to headache pathophysiology. Our in vitro electrophysiological studies found that conditioned media was capable of sensitizing retrogradely-labeled dural afferents. Prior studies in our laboratory showed similar sensitization of dural afferents using direct application of IL-6 to these neurons [40]. Thus, the IL-6 present in conditioned media may be a primary sensitizing agent released from dural fibroblasts. We also found that application of fibroblast-conditioned media directly to the dura in a preclinical behavioral model of headache produced cutaneous facial and hindpaw allodynia. As described above, these effects are consistent with those observed in our prior studies using direct application of IL-6 to the dura in the same behavioral model where we also found cutaneous facial and hindpaw allodynia. Taken together, these findings are consistent with the hypothesis that dural fibroblasts are capable of contributing to headache pathophysiology.

The use of cultured dural fibroblasts in these studies allowed us to examine the release of mediators from this population of cells in the absence of many other cell types that would normally be present in the dura such as neurons, endothelial cells, and macrophages. We confirmed the identity of the cells in the culture dish as fibroblasts using vimentin staining, but this does not selectively label fibroblasts and will label other mesenchymal cells. However, our staining for other cells types found in the dura resulted in little to no labeling. As a result, our data indicate that the conditioned media collected after LPS stimulation contains mediators released primarily from dural fibroblasts. Although we identified IL-6 as one mediator contained in this media, we cannot rule out the possibility that other factors are present in fibroblast-conditioned media. Cardiac fibroblasts are capable of releasing an array of factors including IL-1, TNF- α , Nitric Oxide, and TGF β [33] and dural fibroblasts may also release many of these factors. Future studies are required to determine whether other factors are present in the conditioned media and how these factors may contribute to activation or sensitization of dural afferents.

The stimulus used in these studies to condition media from fibroblast cultures was LPS. As LPS is a component of the cell wall of gram-negative bacteria, the results of our current studies are most applicable to bacterial infection of the meninges such as in meningitis. The findings are nonetheless important as headache is one of the primary symptoms of

meningitis and the mechanisms described here may contribute to the development of headaches during meningitis. Studies have also shown the presence of LPS in the plasma of some migraine without aura patients [6], possibly due to changes in intestinal barrier permeability that allow gastrointestinal flora to leak into the bloodstream, but whether this is a contributing factor to migraine in general is not clear. Regardless, LPS is commonly used as an activator of toll-like receptor 4 (TLR4), a pattern-recognition receptor that responds to pathogen-associated molecular patterns such as those present on invading bacteria. TLR4 is expressed on numerous cell types including immune cells (dendritic cell, monocyte and macrophage, B cells etc), epithelial cells, sensory neurons [1; 5; 8; 9; 12; 15] and has been shown in prior studies to be expressed on fibroblasts from other tissue origins such as those found in adventitia, lung, gingiva, and synovium [16; 17; 35; 37]. In addition to LPS, there are many endogenous ligands for TLR-4 such as the heat shock protein Gp96, fibrinogen, surfactant protein-A, high mobility group box 1 protein (HMGB1) and mRNA [31]. Interestingly, HMGB1 was recently found to be released by cells in the cortex during spreading depression [20]. Although it is unlikely that HMGB1 would migrate to the dura, these studies demonstrate that endogenous processes linked to migraine can be a source of TLR4 activators. There may be other endogenous mechanisms activating TLR4 that initiate the release of IL-6 and other mediators from dural fibroblasts and contribute to headache pathophysiology in the absence of bacterial infection.

Perhaps more relevant to migraine is the question of whether dural fibroblasts release mediators that activate or sensitize afferent nociceptors in response to events that may occur within the dura before or during a migraine attack. Although the pathophysiology leading to migraine is not clear, one of the most common triggers for migraine is stress [18; 22; 24]. Stress is likely to produce sympathetic outflow into the meninges as the dura is innervated by sympathetic efferents [21]. Stress and the resulting sympathetic outflow into the dura may activate receptors on fibroblasts promoting the release of mediators that contribute to headache. In the cardiovascular system, sympathetic outflow onto cardiac fibroblasts can lead to increased IL-6 production via activation of noradrenergic and purinergic receptors on cardiac fibroblasts [3; 4]. If similar events occur within the dura, this may be a mechanism by which stress contributes to the activation of the trigeminovascular system. Although it is tempting to speculate on a broad role for dural fibroblasts in headache, more studies are required to examine whether the physiology of these cells may indeed contribute to disorders such as migraine.

These results provide the first demonstration that dural fibroblasts play a potential role in headache pathophysiology. Although the direct implications of the findings described here with LPS are limited to bacterial infection of the meninges such as in meningitis, dural fibroblasts may play a role in headache disorders beyond meningeal infection. Future experiments will focus on what mediators are released by dural fibroblasts in addition to IL-6 and whether stimuli other than LPS can promote the release of these mediators. Uncovering the role dural fibroblasts play in physiological and pathological events occurring within the meninges may ultimately lead to novel treatments for headache.

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Figure 1. Phase contrast image of dural fibroblasts in culture 3 days post-plating
Scale bar is 100 μm .

Figure 2. Primary cultures of rat dura are predominantly fibroblasts

Rat dura was removed, cultured, and subjected to immunocytochemistry at 3 days post-plating. Staining procedures resulted in the majority of cultured cells labeled with the fibroblast marker vimentin (2A), but not with GFAP, a marker of glial (Schwann) cells (2B), CD68, a marker of macrophages (2C), or vWF, a marker of endothelial cells (2D). Cell nuclei are stained in all panels with DAPI. Scale bar is 100 μ m.

Figure 3. Conditioned media from LPS-treated fibroblasts induces hyperexcitability of dural afferents

Dural afferents treated with V-CM show few action potentials to any of the ramp stimuli (3A). Dural afferents treated with L-CM show significantly more action potentials (3B). The NC-M group also showed few action potentials in response to ramp stimuli (3C). Action potentials in 3A-C were elicited by 1 s ramp current injections ranging from 0.1 to 0.7 nA in 0.2 nA increments as shown in (3D). Differences in the mean numbers of action potentials among groups were analyzed by comparing the slopes of the lines for each group using linear regression ($F(2,70) = 15.50$, $p < 0.0001$). Dural afferents treated with L-CM (Squares, $n = 6$) showed a significant increase in the number of action potentials compared to V-CM (Circles, $n = 6$) and NC-M (Triangles, $n = 7$).

Figure 4. Dural application of conditioned media from LPS-treated fibroblasts elicits cutaneous allodynia

Withdrawal thresholds to tactile stimuli applied to the face (4A) and the hind-paws (4B) were measured in rats before and immediately after dural application of L-CM (n = 9, Squares), V-CM (n=8, Circles), and NC-M (n=8, Triangles). For both facial and hind-paw responses, two-factor ANOVA indicated a significant effect of both treatment and time of both the face and hindpaws. Facial: treatment $F(2,22) = 8.90$, $p = 0.0015$, time $F(5,110) = 2.42$, $p = 0.04$; Hindpaw: treatment $F(2,22) = 164.63$, $p < 0.0001$, time $F(5,110) = 83.41$, $p < 0.0001$. Withdrawal thresholds to tactile stimuli were measured for 5 hrs and data were converted to area over the time-effect curve (AOC) for face (4C) and hindpaw (4D). A one-factor ANOVA with Bonferroni post-test revealed significantly more allodynia with L-CM compared to V-CM and NC-M in both the face and hindpaws ($F = 9.15$ for facial allodynia and $F = 12.04$ for hindpaw allodynia). Significance between groups was (** $p < 0.001$; *** $p < 0.0005$) for L-CM compared to either V-CM or NC-M.

Figure 5. Dural fibroblasts release IL-6 in response to LPS stimulation

Dural fibroblasts cultures were treated with LPS (5 μ g/ml) or vehicle for 1 hour, washed, fresh media was added, and that media was collected after 5 hours. IL-6 content for each condition was measured by ELISA. The 1 hour condition is the media collected at the end of the 1 hour LPS treatment (i.e. it contains LPS). Media collected for 5 hours following removal of LPS and washing (LC-M) contained significantly more IL-6 compared to VC-M. Mean IL-6 levels were compared by two-way ANOVA for treatment and time ($F(1,10) = 247.22$ for treatment and $F(1,10) = 261.79$ for time). (** $p < 0.001$). The bars for 1 hour L-CM and V-CM as well as the 5 hour V-CM bar are less than 10 pg/mL IL-6 and cannot be seen in the graph.