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A novel method for modeling facial allodynia associated with migraine in awake and freely moving rats

Julie Wieseler^{*}, Amanda Ellis, David Sprunger, Kim Brown, Andrew McFadden, John Mahoney, Niloofar Rezvani, Steven F. Maier, and Linda R. Watkins

Department of Psychology & Center for Neurosciences University of Colorado at Boulder Boulder, CO 80309-0345 USA

Abstract

Migraine is a neurovascular disorder that induces debilitating headaches associated with multiple symptoms including facial allodynia, characterized by heightened responsivity to normally innocuous mechanical stimuli. It is now well accepted that immune activation and immune-derived inflammatory mediators enhance pain responsivity, including in the trigeminal system. Nociceptive ("pain" responsive) trigeminal nerves densely innervate the cranial meninges. We have recently proposed that the meninges may serve as a previously unidentified, key interface between the peripheral immune system and the CNS with potential implications for understanding underlying migraine mechanisms. Our focus here is the development of a model for facial allodynia associated with migraine. We developed a model wherein an indwelling catheter is placed between the skull and dura, allowing immunogenic stimuli to be administered over the dura in awake and freely moving rats. Since the catheter does not contact the brain itself, any proinflammatory cytokines induced following manipulation derive from resident or recruited meningeal immune cells. While surgery alone does not alter immune activation markers, TNF or IL6 mRNA and/or protein, it does decrease gene expression and increase protein expression of IL-1 at 4 days after surgery. Using this model we show the induction of facial allodynia in response to supradural administration of either the HIV glycoprotein gp120 or inflammatory soup (bradykinin, histamine, serotonin, and prostaglandin E2), and the induction of hindpaw allodynia in our model after inflammatory soup. This model allows time and dose dependent assessment of the relationship between changes in meningeal inflammation and corresponding exaggerated pain behaviors.

Keywords

migraine; pain; gp120; inflammatory soup; rat; facial allodynia; mechanical allodynia; referred pain; trigeminal

Introduction

Migraine headaches are debilitatingly painful and poorly managed. Migraines are commonly associated with facial allodynia (perception of pain in response to normally innocuous stimuli)

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*Corresponding Author: Dr. Julie Wieseler Department of Psychology & Neuroscience Campus Box 345 University of Colorado at Boulder Boulder, CO 80309-0345 Tel: 303.735.2295 Fax: 303.492.2967 wieseler@colorado.edu.

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(Burstein et al., 2005; Vickers and Cousins, 2000), and corpalgia (extracephalic allodynia) (Cuadrado et al., 2008). In humans, migraines can be halted if treatment with pharmacotherapies such as triptans begins prior to the onset of facial allodynia. If treatment is delayed until after facial allodynia occurs, then treatments fail (Burstein and Jakubowski, 2004). The mechanisms and mediators underlying migraine-associated facial allodynia are largely unknown. A better understanding of this phenomenon may provide better therapeutic targets for the treatment of migraine.

The meninges are commonly thought of as a protective membrane surrounding the brain and spinal cord. However, the meninges surrounding the brain are also innervated by trigeminal nociceptive fibers in close proximity to dense populations of resident immune cells. Indeed the meningeal immune population includes fibroblasts, macrophages, dendritic cells, and mast cells (Artico and Cavallotti, 2001; Braun et al., 1993; Fischer and Reichmann, 2001; McMenamin, 1999; Mercier and Hatton, 2004; Rozniecki et al., 1999; Zenker et al., 1994), each of which are capable of releasing proinflammatory, neuroexcitatory mediators.

Inflammatory soup, composed of a mixture of prostaglandin E₂, serotonin, bradykinin, and histamine, has frequently been used in the migraine literature to study activation of nociceptors in response to immune-derived products commonly released at sites of inflammation, including the meninges. Administration of inflammatory soup (Burstein et al., 1998; Steen et al., 1995) over the meninges induces expansions of the mechanical receptive fields in the skin as well as sensitization of the nociceptors to mechanical stimulation, and sensitization of trigeminal nucleus caudalis neurons in anesthetized animals (Burstein et al., 1998).

The meninges have also been recently implicated in pain processing at spinal levels. Peri-spinal administration of immune products is known to induce mechanical allodynia (Watkins et al., 2001). One means of inducing the release of these immune products is by intrathecal administration of HIV-1 gp120 which influences meningeal immune cells in addition to spinal cord. Indeed, intrathecal gp120 upregulates gene expression and the release of proinflammatory cytokines from the spinal cord meninges (Wieseler-Frank et al., 2007). While the effect of gp120 over the cranial meninges on facial allodynia has not previously been considered, it provides a novel approach for inducing an endogenously released mixture of immune-derived cranial meningeal inflammatory mediators in contrast to administering a pre-set mixture of a subset of such products.

While the inflammatory soup model has been successfully used to elucidate the mechanistic relationship between meningeal nociceptor activation by immune mediators and consequent changes in trigeminal sensory processing, one constraint on the way this model has classically been employed is that the studies, by necessity, have almost exclusively been carried out in anesthetized animals. To extend the classic inflammatory soup method so to allow facial allodynia to be assessed in a freely moving animal, we have developed a new model by which one can easily administer immunogenic stimuli supradurally in awake animals. Others that have also developed methods by which to administer supradural inflammatory soup to awake animals, include Malick et al. (2001) to assess the effects of inflammatory soup on food intake, Oshinsky et al. (2007) to assess facial allodynia following repeated inflammatory soup administration to model episodic headache, and Edelmayer et al. (2009) to assess facial allodynia in response a single administration of inflammatory soup and corresponding treatment with commonly used migraine therapeutics. Even though we are not the first to consider administration of inflammatory soup to awake and freely moving rats, we are the first to present the current method for supradural delivery. We developed our method also to assess facial allodynia here, and beyond that to better study how peripheral immune challenge in the meninges alters trigeminal glial activation (ms in preparation). Our method places bilateral PE10 catheters supradurally, with the tip ending 1mm anterior of lambda. Our goal was to

design a catheter by which we could administer substances over the same area shown to result in neuronal activation in the trigeminal nucleus caudalis (Sp5C). This method allows for the assessment of changes induced by supradurally injected substances without the influence of anesthetics as it is well established that anesthetics can interfere with immune, glial and/or neuronal function that may be important for the phenomenon under study.

Materials and Methods

Subjects

Male Sprague-Dawley rats (250–300 gm; Harlan Sprague-Dawley, Indianapolis, IN) were pair-housed with food and water available *ad libitum*. Room temperature was maintained at $24 \pm 1^\circ\text{C}$ on a 12 hr light: 12 hr dark cycle. Rats were allowed a minimum of 1 wk to habituate to the colony room prior to surgery and testing. Following surgery, animals were singly housed to protect the supradural catheters. For each of the different aspects of methods development, $n=2-5$, whereas for experimental endpoints, $n=6$ per group. The University of Colorado Institutional Animal Care and Use Committee approved all protocols.

Catheters: Choice of materials and preparation

Several considerations defined the final choice of the material used for the supradural catheters. The first goal was to insert the catheters under the skull without piercing the dura. As described below (see “Surgery for catheter placement”), this was partially achieved by appropriate preparation of the skull to enable the catheter to be introduced parallel to, and in the space between, the skull and dura. The diameter of the catheter markedly affected successful placement without dural injury as stretching candidate tubing (such as polyurethane) to minimize its external diameter greatly increased the prevalence of dural piercing and consequent subdural placement of the inserted catheters. A second goal was to place the catheter such that the tip would be terminating just above the occipital lobe, approximately 1 mm rostral of lambda consistent with the literature showing that application of inflammatory soup in this region leads to neuronal activation in the trigeminal nucleus caudalis (Burstein et al., 1998). Relatedly, the proximal tip of the inserted catheter needed to be sufficiently far under the skull entry point to ensure no backflow of injected fluids out of the drill hole through which the catheter was inserted. Thus the catheter material needed to be of sufficient rigidity to allow its placement several mm into the constrained space between skull and dura. To prevent backflow, and hence loss, of injected fluids, the catheter was inserted 6 mm under the skull and then pulled back 2 mm. This procedure resulted in 4 mm of the catheter remaining permanently secured under the skull with a 2 mm supra-dural pocket beyond its internal tip. This combination of distance under the dura plus creation of a supradural pocket successfully retained the volumes (6 μl) injected. The third goal was to secure the catheters in place with a simple, effective method that minimized time and later problems with infection or catheter loss. After trying various procedures, including dental cement and skull screws, it was determined that the most effective method was to hold the catheters in place with superglue. This approach was predicated on a snug fit between the catheter and surrounding skull. This was accomplished by careful choice of drill bit so that the trough created in the skull (see “Surgery for catheter placement”, below) closely apposed the external catheter after insertion.

In the process of designing the optimal catheter, a variety of catheter materials were explored, including silastic, polyethylene and polyurethane tubing of various diameters. Given the tight space between the skull and the dura, it was assumed that reducing the size of the catheter diameter and/or its rigidity would be less traumatizing to the tissue. For this reason, silastic was tested as well as polyurethane as these are by far more pliable than the polyethylene. The flexibility of silastic made it unwieldy to insert under the skull. The polyurethane catheters were also difficult to place due to its pliable nature. While stretching the polyurethane tubing

made it easier to thread, it also dramatically increased instances of piercing the dura, as noted above. The successful method used polyethylene 10 (PE-10) tubing as it is received from the manufacturer.

To prepare catheters, PE10 tubing (BD, Franklin Lakes, NJ) was cut in 6–8 cm lengths. Prior to the day of surgery, catheters were filled with sterile saline and heat-sealed at both ends such that no air bubbles were in the catheter. Catheters were stored in 75% ethanol for 24 hr prior to surgery. Before implantation, catheters were transferred to sterile saline and marked with permanent marker at 4 and 6 mm from the end of the catheter that would be threaded under the skull during surgery.

Surgery for catheter placement

Rats were anesthetized under isoflurane anesthesia and placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). All surgical tools were sterilized in a glass bead sterilizer before and during the surgical procedure and care was taken to keep the surgery environment as clean as possible. The surgical site was shaved and topical Betadine was liberally applied to the skin.

A 2 cm incision was made to expose bregma, and 3% hydrogen peroxide was applied to the surface of the skull to minimize bleeding. The skin was retracted through the use of bilateral weighted clips (Kopf Instruments, Tujunga, CA). A handheld electric drill was used with a Dremel #107 engraving cutter bit to bore two 8–10 mm long troughs in the skull, bilateral to the midsagittal suture. These troughs were each approximately 2 mm wide and positioned parallel to the midsagittal suture and 3–4 mm lateral to it. Care was taken not to penetrate the dura during the drilling process. Troughs were drilled such that the dura was exposed 1 mm caudal to bregma and the shallowest end of the trough was 4–7 mm rostral to bregma (Figure 1).

Once the bilateral troughs were drilled, the catheters were carefully inserted horizontally along the troughs at the point 1 mm caudal to bregma. The catheters were moved between the 4 and 6 mm marks under the skull to create a pocket between the skull and the meninges. The catheters were then glued in place at the 4 mm mark with Superglue (cyanoacrylate) and allowed to dry for approximately fifteen minutes. The external seal of each catheter was then clipped and the catheters flushed with 5 μ l of sterile saline through a glass 50 μ l Hamilton syringe, being careful not to introduce any air into the catheter so to avoid later catheter occlusion by clots. Upon completion of this flush, the external tips of the catheters were re-sealed with heated forceps. The scalp wound was sealed with 9 mm stainless steel wound clips, and powdered Polysporin antibiotic (Pfizer, New York, NY) liberally applied to the surgical site. The rats recovered in a heated recovery box and were then individually housed once ambulatory.

Injection via the pre-implanted catheter

After establishing the methods for secure supradural catheter placement, the next step was to optimize methods for administering solutions via the catheter. As noted above, after placing and securing the catheter, it was flushed with saline. This flush at the time of surgery was instituted to obviate clogging problems encountered when this step was not done. Prior to including this flush procedure, there was a high rate of clogged catheters when injections were attempted 7, 10 and 14 days post surgery. Indeed, it was found that the catheters were reliably clogged if the injection was delayed beyond 4–5 days post surgery. Flushing the catheter with saline on days 4 or 5 after surgery failed to resolve the high incidence of clogged catheters at later time points. As an attempt to prevent clogging, 2–3 μ l heparin (300–1000 units/ml) followed by 1 μ l saline was flushed through the catheter 48–72 hours following surgery. Saline was again flushed through the catheter 24 hours after heparin injection. Another approach placed catheters filled with heparin 24 hours prior to surgery. Once placed during surgery, the

catheters were then flushed with saline. The heparin did not prevent clogging 5–7 days after surgery. While some of the issues were resolved when all air was prevented from entering the catheter, clogging was still a considerable issue beyond 5 days after surgery. The simple procedure of flushing the catheters with sterile saline at the time of surgery, being sure to avoid any air in the catheters, now provides a success rate of 97% and reliable use of the catheters through at least 10 days after surgery.

Variations in the volume and rate of injection were also explored. Initially, 10 l was injected over 75 sec, consistent with previously published methods for intrathecal injections over the spinal cord (Wieseler-Frank et al., 2007). While this volume and rate were successful, as defined by no efflux and the dura remained, there were two concerns. First, this volume and rate of injection would traumatize the dura, thereby causing behavioral changes due to the injection alone. Second, this volume and rate may result in the solution moving from the pocket around the catheter, traveling back along the outside of the catheter, moving to areas other than that being targeted. The successful volume and rate, which avoided the above concerns was found to be 6 µl over 2 min 14 sec (1µl/ 22.3 sec). All injections were given using Hamilton glass syringes (100 µl) with an infusion pump (Razel Scientific Instruments, INC., Stamford, CT; Model A-99, pump set at 36.9). The injector remained connected to the catheter for 1 min following infusion to allow for diffusion of the solution. The 2 catheters were injected serially, alternating which was injected first across animals. Catheter placement was verified at the completion of all experiments.

Blood Brain Barrier Assessment

Given the skull drilling and placement of the catheter into the supradural space, it is possible that these procedures may have been sufficient to disrupt blood brain barrier permeability at the time of drug testing. Potential increases in blood brain barrier permeability were examined by intravenously administering Evans blue dye, which selectively binds albumin in the bloodstream. Wherever the blood brain barrier is intact, Evans blue dye does not enter the brain parenchyma, making exclusion of the dye a widely used test for blood brain barrier integrity (Sharma and Johanson, 2007). Thus, 4 days post surgery, the point at which behavioral effects of administered supradural drugs would normally be tested, rats were anesthetized (isoflurane) and their right jugular veins exposed. The Evans blue dye (12.5 mg/ in 500 µl saline) was injected, via brief jugular catheterization (PE50), and then the right jugular and overlying skin were closed. Rats were returned to their home cages and allowed to recover for 2.5 hrs, at which point the rat was overdosed with sodium pentobarbital and transcardially perfused with saline. Following perfusion, the brain was placed in a 4% paraformaldehyde solution and stored at 4°C overnight. The brain was then transferred to 30% sucrose solution and stored at 4°C until sectioned in a cryostat. After freezing, tissue was sectioned coronally at 60 µm beginning immediately beneath the catheter tip site, and then 1 cm anterior and posterior. Following visualization of tissues, Evans blue dye was found in the dura, as expected, as the dura is outside of the blood brain barrier; however, dye was not found anywhere in the brain parenchyma.

Pain Assessment

Facial allodynia—Facial allodynia was assessed via von Frey testing. All testing was performed blinded with respect to group assignment. Multiple regions on the face as well as regions beyond the face were initially tested to explore the extent of regional involvement. The following areas were assessed bilaterally: 3 areas around the eye (immediately below, immediately lateral, 2 mm lateral), the cheek, the shoulder, the forearm, and the dorsal aspect of the forepaw. Allodynia by definition is a heightened sensitivity to tactile stimuli, thereby leading to a very uncomfortable, and painful, state. Here a response was counted when the animal moved away from the von Frey stimulus, or moved the stimulus away by paw swipe. Among the regions tested, mechanical allodynia was only observed in those regions tested

around the eye, consistent with the first branch of the trigeminal nerve (V_1). Two methods accepted in the literature for assessing mechanical allodynia are to 1) test a gram force filament in the middle of the logarithmic range and then increase or decrease the filament size until the animal reliably responds (Ren, 1999); and 2) find a gram force filament that the majority of naïve rats respond to, and a gram force filament that the majority of allodynic rats respond to (Tawfik et al., 2007). Both methods were explored. With the first method, each filament was left in contact with the skin for 2 sec. Using the second method, the skin was exposed to the filament briefly. These procedures are in accordance with prior literature (Ren, 1999; Tawfik et al., 2007).

The initial data below with gp120 was collected using the Tawfik et al. (2007) method because the primary question here was simply whether a known immune activator, gp120, was capable of inducing facial allodynia. While this early study successfully used the Tawfik method to document facial allodynia by gp120, the remainder of the studies described here changed the behavioral assessment method so to allow the determination of absolute thresholds, which the Tawfik method cannot do. Thus, the following studies measured behavior via a modified method of Ren (1999). Briefly, each rat was habituated to a leather glove, and encouraged during habituation to stay in the glove. Following 5 days of general habituation to handling (~5 min/day) and two 5 min habituation sessions to the glove, baseline behavior was assessed. A logarithmic series of 10 calibrated Semmes-Weinstein monofilaments (von Frey hairs; Stoelting, Wood Dale, IL) were applied to both the left and right periorbital region. Log stiffness of the hairs is determined by $\log_{10}$ (milligrams $\times$ 10). Starting at the smallest filament size used here (log stiffness value, 4.31; 2 grams), a stepwise progression was followed to the maximum filament size used here (log stiffness value, 6.45; 180 grams), or until the rat responded 5 times to a filament. The rat was stimulated 5 times with each filament for 2 seconds on each side of the face, alternating sides, and separating stimulations by approximately 3 seconds. These data were then analyzed as previously described (Milligan et al., 2000).

Hindpaw allodynia—Absolute thresholds were determined in the sciatic innervation area of the hindpaws, using the up-down method with a series of calibrated filaments (von Frey test), as previously described in detail (Milligan et al., 2000). All testing was performed blinded with respect to group assignment. Briefly, the 10 calibrated monofilaments (von Frey hairs) were applied to both the left and right hindpaws in random order to determine the stimulus intensity threshold stiffness required to elicit a paw withdrawal response. The 10 stimuli had the following log-stiffness values ranging from 3.61 (0.407 g) up to 5.18 (15.136 g).

Absolute threshold calculation—This range of monofilaments produces a logarithmically graded slope when interpolating a 50% response threshold of stimulus intensity [expressed as $\log_{10}$ (milligrams $\times$ 10)]. Assessments were made before (baseline) and at specific times after surgery or application of inflammatory soup or gp120, as detailed in the studies below. The behavioral responses were used to calculate the 50% paw withdrawal threshold (absolute threshold) by fitting a Gaussian integral psychometric function by using a maximum-likelihood fitting method. This fitting method allows parametric statistical analyses (Harvey, 1986; Treutwein and Strasburger, 1999).

Preparation of gp120 and inflammatory soup

Frozen solutions of recombinant gp120 (product 1021, lot number 2S3/1.5; from ImmunoDiagnostics, Inc.) were thawed, aliquoted at 1 μ g/ μ l, and stored at -75°C . Vehicle, 0.2% rat serum albumin (Accurate Chemical and Scientific Corp, Westbury, NY) in PBS, was aliquoted and stored at -75°C . The gp120 was endotoxin free (documented by manufacturer) and handled using sterile technique. Frozen aliquots of gp120 and vehicle were thawed immediately before administration, and gp120 was diluted to concentrations specified in each

experiment, in a final solution containing 0.1% rat serum albumin to facilitate gp120 administration. We have previously shown that the rat serum albumin does not interfere with the actions of gp120 (Milligan et al., 2000). Aliquots were kept on ice during use and discarded within 30 min.

Inflammatory soup (1 mM bradykinin, serotonin, and histamine, and 0.1 mM prostaglandin E₂, or 2 mM bradykinin, serotonin, and histamine, and 0.2 mM prostaglandin E₂; pH 5.5) (Steen et al., 1995) is made fresh just prior to use from stock solutions. Stock solutions are stored at 4°C for a maximum of 30 days.

Assessment of dura changes in response to indwelling catheter and injection via ELISA and PCR

Enzyme Linked Immunosorbant Assay (ELISA)—Meningeal tissue was collected from catheterized rats (2 × 4 mm piece of tissue surrounding the catheter) and naïve rats (2 × 4 mm piece of tissue comparable to that collected from catheterized rats) for cytokine analyses. Cytokines in these meningeal samples were measured using commercially available ELISA kits for rat IL-1 β and rat IL-6 (R&D Systems, Minneapolis, MN). The ELISAs were performed according to manufacturer's instructions and to our published method for carrying out the ELISAs in serial (O'Connor et al., 2004). Prior to being assayed, meninges were dissociated by sonication buffer containing 5% fetal calf serum and an enzyme inhibitor cocktail consisting of 100 mM amino-*N*-caproic acid, 10 mM EDTA, 5 mM benzamidine-HCl, and 0.2 mM phenylmethylsulfonyl fluoride (all from Sigma, St. Louis, MO). Sonicated samples were centrifuged (10,000 rpm, 10 min, 4°C), and supernatants were removed and assayed immediately. The total protein concentration was determined by the Bradford protein assay.

Real-time reverse transcription-PCR (RT-PCR)—In order to assess changes in the resident meningeal immune cells as a result of the surgery itself, meningeal tissue was collected from catheterized rats and naïve rats as described for protein measures, above. Tissues were collected 4 days post surgery, the same time point post surgery when immunogenic stimuli are administered. Tissues were processed for gene expression using real-time RT-PCR. To initially characterize how the meningeal immune cells change in response to injection of the different manipulations, we compared meningeal tissue among animals receiving a single or repeated (2 injections separated by 2 hours) administration of saline, inflammatory soup, or gp120, and tissue collected 2 hours post injection. This time point was chosen as that is 1) when the second injection is administered following the first, so is indicative as to the state of the cells at that point, and 2) when behavior becomes reliably robust following the second injection. Genes analyzed were for the general innate immune cell marker, CD11b, commonly used to identify macrophages, monocytes, and neutrophils (Ross and Vetvicka, 1993); CD68 is a marker found on macrophages, monocytes, neutrophils, as well as dendritic cells and mast cells (Gordon, 1999); and, major histocompatibility complex II (MHCII), a marker that is often considered to be indicative of a cell that maybe primed to respond robustly to future stimulation, but is not currently in an activated state (Lambert and Paulnock, 1989; Mosser, 2003).

RNA isolation and enrichment—Total RNA was isolated based on the methods Chomczynski and Sacchi (Chomczynski and Sacchi, 1987), as previously described (Johnston et al., 2004). Samples were DNase treated (DNA-free kit; Ambion, Austin, TX).

cDNA synthesis—Total RNA was reverse transcribed using the SuperScript II First Strand Synthesis system for reverse transcription (RT)-PCR (Invitrogen), per manufacturer's instructions and as previously described (Johnston et al., 2004). cDNA samples were diluted twofold in DNase free water and stored at -20°C.

Primer specifications—cDNA sequences for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH; accession number M17701) were obtained from GenBank at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). Primer sequences for rat GAPDH (forward: 5'-GTTTGTGATGGGTGTGAACC-3'; reverse: 5'-TCTTCTGAGTGGCAGTGATG-3'; 162 bp product), CD11b (Complement Receptor 3) (forward: 5'-CTGGGAGATGTGAATGGAG-3'; reverse: 5'-ACTGATGCTGGCTACTGATG-3'; 114 bp product), MHCII (forward: 5'-AGAGACCATCTGGAGACTTG-3'; reverse: 5'-CATCTGGGGTGTGTTGGA-3'; 130 bp product), CD68 (forward: 5'-AGACGACAATCAACCTACCA-3'; reverse: 5'-AGACAGAGAAGGGAGATAAC-3'; 124 bp product), TNF (forward: 5'-CTTCAAGGGACAAGGCTG-3'; reverse: 5'-GAGGCTGACTTTCTCCTG-3'; 87 bp product), IL-1 (forward: 5'-GAAGTCAAGACCAAAGTGG-3'; reverse: 5'-TGAAGTCAACTATGTCCCG-3'; 130 bp product), and IL-6 (forward: 5'-ACTTCACAGAGGATAACCAC-3'; reverse: 5'-GCATCATCGCTGTTTCATAC-3'; 143 bp product) were designed using the Oligo Analysis and Plotting Tool (Qiagen, Valencia, CA; oligos.qiagen.com/ooligos/toolkit.php?) and tested for sequence specificity using the Basic Local Alignment Search Tool at NCBI. Primer specificity was further verified by melt curve analysis (see below).

Quantitative real-time PCR—PCR amplification of cDNA was performed using the Quantitect SYBR Green PCR kit (Qiagen), as previously described (Johnston et al., 2004). cDNA (1 µl) was added to a reaction master mix (25 µl) containing SYBR Green I, fluorescein (10 nM), and gene-specific primers (500 nM each of forward and reverse primer). Reactions were conducted in triplicate in 200 µl thin wall tubes (Bio-Rad). A melt curve analysis was conducted to confirm uniformity of product formation, primer dimer formation, and amplification of nonspecific products.

Real-time detection and quantitation of PCR product—Formation of PCR product was monitored in real time using the MyiQ Single-Color Real-Time PCR Detection system (BioRad). Fluorescence of SYBR Green I was captured 72°C. Threshold for detection of PCR product fell within the log-linear phase of amplification for each reaction.

Relative quantitation of gene expression—Relative gene expression was determined using the 2^{-delta delta ct} method, as previously described (Johnston et al., 2004).

Toluidine Blue Mast cell staining

To assess the state of mast cells on day 4 post surgery, dura was collected and stained with the mast cell specific stain, Toluidine blue. Rats were transcardially perfused with physiological saline followed by 2% paraformaldehyde. Consistent with tissue collection for gene and protein expression, 2 × 4 mm meningeal tissue was collected from naïve and catheterized rats. Tissues were directly mounted on gelatin-coated slides, and dried overnight. Tissues were stained with 0.25% acidified toluidine blue for 30 sec.

Injection paradigm and validation of model

To assess the effects of surgery on behavior, responses to facial stimuli were compared between rats: (a) naïve to surgery, (b) that had undergone a sham surgery in which the scalp skin was incised and the skull scraped and cleaned but not drilled, and (c) that had undergone bilateral catheter placement. Behavior was measured over time following surgery.

To experimentally verify the proposed model, immunogenic stimuli (gp120 or inflammatory soup) or vehicles were bilaterally injected via the supradural catheters. A single, bilateral injection of either stimulus did not induce facial allodynia, as measured using von Frey

filaments hourly for 6 hr and again at 24 hr post injection. A second injection 24 hr after the first again did not result in facial allodynia. When the interval was reduced from 24 hr to 2 hr between injections, both gp120 and inflammatory soup induced facial allodynia, as measured using von Frey filaments, thereby validating our model for inducing facial allodynia. No differences were observed between the right and left sides, and because of this only data from the left are presented. Bilateral allodynia was expected, given bilateral infusion of the inflammatory mediators.

To test if supradural administration of inflammatory soup induced exaggerated pain responses beyond the face, hindpaw withdrawal thresholds were assessed using the 2- injection paradigm described above. Following the second injection of 0, 1, or 2 mM of inflammatory soup, hindpaw withdrawal responses were tested 2 hours later, the same point at which facial allodynia was observed. As no differences were expected between right and left hindpaws, data for left and right hindpaws were averaged for analysis.

Statistics

Data following gp120 administration were analyzed using the nonparametric statistic, Wilcoxon signed rank test. This test was chosen because the data collected reflects the number of responses out of 5 stimulations, requiring a nonparametric statistic. All other data was analyzed by either a t-test, one-way ANOVA or two-way ANOVA using GraphPad Prism version 5.00 for Mac OS X (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

Results

Success rate

When first learning this surgery, approximately 16% of rats are excluded from analysis due catheters that are found upon postmortem examination to be misplaced or dislodged/missing (<1%) or to have pierced the dura (~16%). With experience, the success rate rapidly improves to routinely be 0% exclusion (100% success rate).

Changes in meningeal immune cell gene and protein expression

Changes in gene expression for CD11b, MHCII and CD68 were used to assess the activation state of meningeal immune cells in response to surgery, and after single or repeated application of saline, inflammatory soup, and gp120. Surgery was not found to significantly influence meningeal immune cell markers, as no significant differences were revealed with a t-test analysis of mean values for CD11b, MHCII and CD68 gene expression between naïve tissue compared to tissue collected from rats that had undergone catheter placement surgery. The oneway ANOVA comparing means among single and double administration of saline, inflammatory soup, or gp120 reveal differences with respect to CD11b ($F(5,31)=5.029$, $p=0.002$; Figure 3A) and MHCII ($F(5,31)=3.1264$, $p=0.02$; Figure 3B), though no differences were observed with respect to CD68. Tukey's Post hoc analysis of revealed that a single and double application of gp120 significantly up-regulated CD11b gene expression compared to saline, and that a double application of gp120 significantly up-regulated CD11b gene expression compared to inflammatory soup. Further post hoc analysis of MHCII did not reveal significant differences, though the means for a single administration of inflammatory soup or gp120 increased MHCII gene expression.

Meningeal gene and protein expression of the proinflammatory cytokines IL-1, TNF, and IL-6 were analyzed using real-time PCR and ELISA, comparing rats bilaterally catheterized 4 days prior to naïve controls. No statistical differences were revealed when analyzing gene or protein expression for TNF and IL-6. Differences were observed with respect to IL-1. Gene expression

analysis showed that IL-1 mRNA was significantly decreased in meninges of catheterized rats compared to meninges from naïve controls ($t(5) = 3.499$, $p = 0.02$; Figure 2A). In contrast, IL-1 protein was significantly elevated 4-days post surgery, compared to controls ($t(10) = 4.260$, $p = 0.002$; Figure 2B). As performed, this ELISA result cannot differentiate intracellular IL-1 protein content from released IL-1 protein, so no conclusions regarding release can be drawn from these data.

Facial allodynia in response to surgery

Facial response thresholds were compared between surgically naïve controls, sham operated rats (surgery stopping just prior to drilling of the skull) and bilaterally catheterized rats. Behaviors were recorded for all rats across time following surgery for bilateral catheter placement. The two way ANOVA revealed a significant interaction, $F(6,45) = 3.763$, $p = 0.004$. Post hoc analysis revealed that sham operated and catheterized groups differed from the naïve group 1 and 2 days post surgery ($p < 0.05$) but did not differ between themselves. There were no differences among groups on days 3 or 4 (Figure 4).

Facial allodynia in response to supradural gp120

A single injection of 1.75 or 3.5 μg gp120 did not induce facial allodynia, nor did two injections of 3.5 μg gp120 separated by 24 hours. However, two bilateral injections of 1.75 μg gp120 separated by 2 hours did induce facial allodynia, with every rat receiving two injections of gp120 showing increased responsivity to stimuli applied to the face. Animals treated with saline did not respond to the 6 g VonFrey filament whereas animals treated with gp120 were responsive ($W = -15$, $N=10$, $p=0.05$; Figure 6).

Facial and hindpaw allodynia in response to supradural inflammatory soup

A single injection of 1 mM inflammatory soup (0.1 mM prostaglandin E2) did not induce facial allodynia, nor did two injections of the same concentration of inflammatory soup separated by 24 hours. The two-way ANOVA comparing absolute allodynia threshold showed a significant interaction between supradural saline and supradural inflammatory soup across time ($F(2,15) = 3.803$, $p < 0.05$; Figure 7). Bonferroni post hoc tests showed that the two groups did not differ at baseline, but did differ significantly at 2 ($p < 0.01$) and 4 ($p < 0.01$) hours post the second injection, with every rat receiving two injections of inflammatory soup showing increased responsivity to stimuli applied to the face. Anecdotally, animals that displayed facial allodynia following either supradural inflammatory soup or gp120 typically responded more robustly to suprathreshold stimuli than did vehicle controls, but as this was not quantified, no data are presented for this observed difference in behavior.

Hindpaw allodynia was measured in our two-injection paradigm, 2 hours after the second injection. The one-way ANOVA comparing absolute allodynia threshold showed a significant effect of inflammatory soup on hindpaw mechanical allodynia, $F(2,8) = 5.181$, $p < 0.05$. Post hoc analysis showed that 1 and 2 mM concentrations of inflammatory soup induced the hindpaw allodynia compared to saline ($p < 0.05$; Figure 8).

Discussion

We describe here a method for administering solutions to the dura in awake and freely moving rats. We validated our model by demonstrating that supradural application of inflammatory soup or gp120, 2 applications separated by 2 hours, leads to facial allodynia. Additionally, the 2 applications of inflammatory soup induced hindpaw allodynia 2 hours post the second injection. The supradural catheterization allows for direct application of drugs to the microenvironment of interest (meninges) while allowing all the potentially involved constituents (e.g. resident immune cells, skull covering) to respond. Administration of

immunogenic stimuli over the dura activates local nociceptors leading to neuronal activation in the caudal nucleus of the trigeminal complex (Burstein et al., 1998). This activation has been interpreted as modeling facial allodynia associated with migraine headache. Our model allows for the measurement of the behavioral manifestation of this activation.

Our goal was to design a model by which we can characterize and better understand the role of peripheral immune cells (macrophages, mast cells, and dendritic cells) and, in the future, corresponding central immune cells (microglia and astrocytes) in facial allodynia resulting from immune stimulation of the meninges. The surgery procedure alone does not alter behavior by 4 days after surgery. It also does not globally alter the immune state of the meninges present 4 days post surgery; rather, selective changes were observed. Gene expression for the immune activation markers examined were not altered 4 days post surgery; whether protein expression may have changed is as yet unknown as proteins were analyzed by ELISA and appropriate ELISAs for the expression markers examined do not exist. As ELISAs do exist for proinflammatory cytokines, both protein and mRNA could be analyzed. TNF and IL-6 were not affected, at either the mRNA or protein level, by surgery 4 days prior; however, IL-1 gene expression in catheterized rats was lower than that measured in tissue from naïve controls whereas IL-1 protein was elevated by prior surgery. Whether this IL-1 protein increase reflects increased intracellular stores versus release cannot be determined from this analysis. An exploration of mast cell staining revealed that the majority of the mast cells proximal to the catheter were degranulated 4 days after surgery. As only some mast cell components are contained within granules, no conclusions can be made at present regarding other mast cell products. Taken together, these data indicate that the immune state of the meninges after bilateral catheterization is not globally altered, but some specific changes were defined. Notably, there have been no studies published, to date, on any of the other migraine models in awake animals regarding the effects of those surgical procedures on such immune parameters. While it is not clear how they might compare, it would not be untoward to anticipate similar findings. Levy et al. (2007) showed robust mast degranulation when removing large pieces of skull, and found that the nociceptors remained responsive to stimulation with inflammatory soup. In the current model, the degranulation state of the mast cells suggests that mast cell granules are not contributing to facial (or hindpaw) allodynia induced by supradural inflammatory soup (where components of mast cell granules are directly replaced by the mixture applied) or, more interestingly, in response to gp120. The latter observation would be in accordance with prior literature that does not, to date, reveal evidence of mast cell degranulation in response to gp120. Hence, at least these 2 supradural inflammatory agents may be optimal for study using this model.

Our model is the first to test an immunogenic stimulus (gp120) other than inflammatory mediators contained in the commonly used inflammatory soup (serotonin, bradykinin, histamine and prostaglandin E2). Further we show here that, indeed, application of either inflammatory soup or gp120 activates the meningeal resident immune cells. Using our model we show induction of facial allodynia following just 2 injections separated by 2 hours of either inflammatory soup or gp120. The fact that 2 successive injections are required spaced by 2 hours for sufficient immune activation, indicating that the first injection appears to prime the cells to respond to future stimulation is evident in the behavior as well as the gene expression. In addition to inducing facial allodynia, our 2-injection paradigm of inflammatory soup induced hindpaw allodynia as well. A single injection of either stimulus did not induce facial allodynia, whereas a second injection induced facial allodynia, and at the time behavior is robust, elevations in MHCII are also present. We are the first group to show specific changes in meningeal immune activity that corresponds with the expression of facial allodynia, and corresponding induction of hindpaw allodynia, in awake and freely moving rats.

We are not the first group to design a model to measure behavioral responses to supradural inflammatory soup in awake and freely moving rats (Edelmayer et al., 2009; Malick et al., 2001; Oshinsky and Gomonchareonsiri, 2007). However, we are the first to do so using the methods described here. Our method places catheters bilaterally, allowing for the consideration of lateral meningeal involvement in activating the trigeminal nucleus caudalis. The other groups considering supradural inflammatory soup in awake and freely moving rats placed a single cannula either directly over the transverse sinus and lateral to the superior sagittal sinus, directly over the intersection of the transverse sinus and the superior sagittal sinus, or 1 mm left of midline and 1 mm anterior to bregma. We use superglue to hold our catheters in place in contrast to skull screws and dental cement, which we tested and eliminated due to potential inflammatory issues compromising the model compared to the superglue/tight channel method described here. The use of skull screws and dental cement required further drilling in the skull, thereby further traumatizing this tissue. Our infusion method is also unique. In our model, our animals are handled during supradural catheter injection, the whole process being completed within 8 min. The total volume for each side injected here is 6 μ l of 1 mM inflammatory soup (0.1mM prostaglandin E2), in contrast to 20 μ l of the same concentration in the Oshinsky model or 10 μ l of 2 mM inflammatory soup (0.2 mM prostaglandin E2) in the Edelmayer model. The model and injection paradigm described here result in observable behavioral changes within 2 hours of the second injection versus 1 hour post a single infusion in the Edelmayer model or 5 infusions separated by 48–72 hours for the Oshinsky model. An additional difference between our model and that of Edelmayer et al. is the testing procedure. Edelmayer et al. (2009) tested facial allodynia by assessing responses to von Frey filaments applied to the midline of the forehead at the level of the eyes. We tested facial allodynia by assessing responses to von Frey filaments applied to the periorbital regions around the left and right eye, similar to Oshinsky et al. (2007).

Much of the work done in the last several years testing the effect of inflammatory soup has been done in anesthetized animals (Jakubowski et al., 2005; Jakubowski et al., 2007; Levy et al., 2008; Oshinsky and Gomonchareonsiri, 2007; Oshinsky and Luo, 2006). The method is such that a significant portion of the skull is acutely removed, necessitating the use of anesthesia. This anesthetized rat model has laid the groundwork for understanding the mechanisms within the meninges, including resident immune cell activity, that result in activation of nociceptors (Levy et al., 2007). One of the drawbacks of the original model is that it does require anesthetic. Many of the mechanisms identified to be responsible for modulating exaggerated pain states cannot be adequately studied in an anesthetized animal. Commonly used anesthetics including isoflurane, halothane, and sodium pentobarbital all affect immune cell and glial activity, beyond their well known suppression of neuronal activation (Itoh et al., 2004; Kudo et al., 2001; Martin et al., 1995; Roughan and Laming, 1998; Toda et al., 2008; Wentlandt et al., 2006; Wise-Faberowski et al., 2006). Thus these mediators cannot be accurately studied in anesthetized animals when immune and/or glial mediators are suspected as potential mediators.

A significant difference between our model and those with anesthetized animals, and, as described above, those by Oshinsky et al. (2007) and Edelmayer et al. (2009), is the volume of inflammatory soup used. These differences may explain, in part, why our model requires two applications to show reliable facial allodynia. Our method differs from the traditionally used method in that it uses a much smaller volume of inflammatory soup. With anesthetized models, the method involves bathing the meninges with the inflammatory stimulus. Using a smaller volume of inflammatory soup over a more discrete dural area (3 mm $\times$ 3 mm area vs. dorsal surface of the left hemisphere and transverse sinus), we have found that two administrations are necessary to induce facial allodynia, such that there are 2 injections of 6 μ l / side in contrast to flooding the dura exposed by craniectomy. While Edelmayer et al. (2009) reported facial allodynia after using a single administration of inflammatory soup, they

used was twice the concentration of inflammatory soup as used here and it was administered at a greater volume. Use of our model, with its 2-injection (separated by 2 hours) paradigm, leads to reliable facial allodynia similar to that reported by migraine patients.

In summary, we have presented here a new model for the study of dural inflammation and resulting behavioral evidence of the induction of facial allodynia and extracephalic allodynia. Furthermore, inducing dural inflammation in awake and freely moving animals provides a better forum to more thoroughly characterize underlying biochemical mechanisms influencing corresponding behavior. We show here facial allodynia following supradural administration of both the inflammatory soup and the immunogenic stimulus, gp120, in awake and freely moving animals. This model can be effectively used to expand previous findings in anesthetized animals by revealing the behavioral manifestation of the pain response.

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Figure 1. Photograph of the supradural catheter implantation sites

Troughs are bilaterally drilled in the skull beginning 2-mm from midline. The troughs are approximately 4-mm long, becoming gradually deeper at the more caudal point. At this point the skull is pierced, while leaving the dura intact. The catheters, constructed from PE-10 tubing, are guided by the troughs and gently inserted bilaterally between the skull and dura mater, with the gently sloping troughs allowing the catheter to slip beneath the skull parallel to the dural surface so to avoid piercing the dura. The catheters are marked to indicate when each is inserted 4–6 mm.

Figure 2. Gene and Protein expression for IL-1 are differentially altered in response to surgery
A) IL-1 gene expression was lower compared naïve control tissues. **B)** IL-1 protein expression was elevated compared to naïve control tissues. Surgery significantly influences the proinflammatory state in the meninges.

Figure 3. Gene expression is altered in response to saline, inflammatory soup and gp120

A) A single application of gp120 (gp120(1)) and double application of gp120 (gp120(2)) significantly up-regulated CD11b gene expression compared to single or double application of saline (saline (1) or saline (2); *), and compared to a single application of inflammatory soup (IS (1); **). **B)** MHCII expression was also affected, however the post hoc analyses did not reveal further significant differences. The graph suggests that single application of inflammatory soup (IS (1)) or gp120 (gp120(1)) up-regulates MHCII gene expression whereas saline, single or repeated application (saline (1) or saline (2)) does not alter MHCII.

Figure 4. Facial allodynia induced by surgery is resolved 4 days post

Surgery for the placement of bilateral supradural catheters and sham surgery (skin incision with skull scraping) induced facial allodynia immediately following surgery on days 1 and 2. By day 4, behavior among surgery groups and naïve to surgery rats were indistinguishable. * indicates differences compared to naïve.

Figure 5. Mast cells are degranulated 4 days post supradural catheterization

A) Naïve meningeal tissues comparable to those surround the supradural catheter, and B) meningeal tissue surrounding the supradural catheter, stained with mast cell specific stain, toluidine blue.

Figure 6. Development of facial allodynia in rats injected supradurally with gp120

Following baseline (pre-drug) assessment of response to calibrated von Frey filaments, rats received supradural injections of either saline or gp120. Two successive supradural injections of saline produced no reliable changes in behavior of awake and freely moving rats across the timecourse tested. In contrast, two successive supradural injections of gp120, separated by 2 hours, induced facial allodynia at 3 hours after the second injection.

Figure 7. Development of facial allodynia in rats injected supradurally with inflammatory soup (IS)

Following baseline (pre-drug) assessment of response to calibrated von Frey filaments, rats received supradural injections of either saline or IS. Two successive supradural injections of saline (filled circles) produced no reliable changes in the behavior of awake and freely moving rats across the timecourse tested. In contrast, two successive supradural injections of IS, separated by 2 hours, induced facial allodynia at 2 and 4 hours after the second injection.

Figure 8. Development of hind-paw allodynia in rats injected supradurally with inflammatory soup (IS)

Following baseline (pre-drug) assessment of response to calibrated von Frey filaments, rats received 2 successive supradural injections of saline, 1, or 2 mM IS. Based on facial allodynia time points, hind-paw allodynia was assessed 2 hours post the second injection. IS induced hind-paw allodynia at both 1 and 2 mM concentrations. * indicate differences from saline.