Transcriptional Changes in Dorsal Spinal Cord Persist after Surgical Incision Despite Preemptive Analgesia with Peripheral Resiniferatoxin

Stephen J. Raithel, B.A., Matthew R. Sapio, Ph.D., Danielle M. LaPaglia, B.A., Michael J. Iadarola, Ph.D., Andrew J. Mannes, M.D.

ABSTRACT

Background: Peripheral nociceptors expressing the ion channel transient receptor potential cation channel, subfamily V, member 1, play an important role in mediating postoperative pain. Signaling from these nociceptors in the peri- and postoperative period can lead to plastic changes in the spinal cord and, when controlled, can yield analgesia. The transcriptomic changes in the dorsal spinal cord after surgery, and potential coupling to transient receptor potential cation channel, subfamily V, member 1–positive nociceptor signaling, remain poorly studied.

Methods: Resiniferatoxin was injected subcutaneously into rat hind paw several minutes before surgical incision to inactivate transient receptor potential cation channel, subfamily V, member 1–positive nerve terminals. The effects of resiniferatoxin on postincisional measures of pain were assessed through postoperative day 10 (n = 51). Transcriptomic changes in the dorsal spinal cord, with and without peripheral transient receptor potential cation channel, subfamily V, member 1–positive nerve terminal inactivation, were assessed by RNA sequencing (n = 22).

Results: Peripherally administered resiniferatoxin increased thermal withdrawal latency by at least twofold through postoperative day 4, increased mechanical withdrawal threshold by at least sevenfold through postoperative day 2, and decreased guarding score by 90% relative to vehicle control ($P < 0.05$). Surgical incision induced 70 genes in the dorsal horn, and these changes were specific to the ipsilateral dorsal horn. Gene induction with surgical incision persisted despite robust analgesia from resiniferatoxin pretreatment. Many of the genes induced were related to microglial activation, such as Cd11b and Iba1.

Conclusions: A single subcutaneous injection of resiniferatoxin before incision attenuated both evoked and nonevoked measures of postoperative pain. Surgical incision induced transcriptomic changes in the dorsal horn that persisted despite analgesia with resiniferatoxin, suggesting that postsurgical pain signals can be blocked without preventing transcription changes in the dorsal horn. **(Anesthesiology 2018; 128:620-35)**

ADEQUATE postoperative analgesia continues to pose a major clinical challenge. Despite the increased focus on pain control in recent decades, a large number of patients report severe pain after surgery with current standard of care postoperative analgesics.[1,2] In addition to causing suffering in the immediate postoperative time period, uncontrolled postoperative pain is a risk factor for chronic postsurgical pain after a variety of surgical procedures.[3–5] Previous studies indicate that long-lasting, multimodal analgesia can reduce primary afferent nociceptor firing during the perioperative period.[6–8] Nonetheless, achieving simple, effective long-duration postoperative analgesia with minimal patient intervention remains elusive, and new analgesic targets are needed to control postoperative pain. Primary afferents that express transient receptor potential cation channel, subfamily V, member 1 (TRPV1) transduce signals from noxious heat and inflammatory stimuli,[9,10] and the activation of these fibers is perceived as painful, making these fibers, as a class, attractive targets to silence in the perioperative period. Indeed, after surgical incision, TRPV1$^+$ afferents have been implicated in heat hyperalgesia and spontaneous afferent activity.[11]

What We Already Know about This Topic

- Nociceptors expressing the transient receptor potential cation channel, subfamily V, member 1, ion channel play important roles in postoperative pain
- Resiniferatoxin is a potent transient receptor potential cation channel, subfamily V, member 1, agonist capable of silencing nerve terminals and providing analgesia lasting weeks

What This Article Tells Us That Is New

- Using a rat model of postsurgical pain, it was shown that a single localized injection of resiniferatoxin reduced evoked and nonevoked pain-related behaviors over the entire period of postoperative recovery
- Analysis of spinal cord dorsal horn tissue revealed that resiniferatoxin pretreatment did not block most changes in gene expression, including the expression of genes associated with microglia, despite reducing pain-related behaviors

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org). This work has been presented in abstract form at the American Pain Society 36th Annual Scientific Meeting in May 2017 in Pittsburgh, Pennsylvania.

 Anesthesiology 2018; 128:620-35

<zdoi;10.1097/ALN.0000000000002006>

Selective and temporary chemoablation of TRPV1+ nociceptors with resiniferatoxin could provide the requisite long-lasting analgesia needed for postoperative pain control. Resiniferatoxin provides highly effective analgesia in multiple preclinical models of pain,[10,12–14] and is in phase I clinical trials for intrathecal use in patients with severe pain associated with advanced cancer. As an ultrapotent analog of capsaicin, resiniferatoxin binds to TRPV1 and causes prolonged channel opening, calcium influx, and cytotoxicity to the neuron terminals.[15,16] As a result, treatment with resiniferatoxin blocks several modalities of nociceptive signaling from nerve endings containing TRPV1 in addition to those signals transduced by the channel itself. A single peripheral administration of resiniferatoxin leads to an analgesic state that lasts between 7 and 14 days,[14] a duration of effect longer than the usual duration of severe postoperative incisional pain.[17] Thus, nociceptive input from TRPV1+ afferents can theoretically be blocked during the entire perioperative period after a single preoperative injection. In this report, we first establish the efficacy of resiniferatoxin in decreasing postincisional pain in rats.

Fewer pain signals entering the central nervous system potentially decrease the likelihood of inducing plastic changes in the central nervous system known as central sensitization, which is a concern after surgery.[7] To date, the transcriptional changes in the dorsal horn (DH) of the spinal cord that occur after surgical incision remain incompletely defined, and so it is unclear to what extent gene expression is altered by peripheral incision. Furthermore, postincisional pain is thought to involve different mediators and mechanisms than purely inflammatory or neuropathic pain, and insight into transcriptomic changes in those models might not be fully applicable to a postsurgical pain model.[18,19] Therefore, we performed RNA sequencing (RNA-Seq) of the dorsal spinal cord to characterize the molecular changes in the DH induced by surgery. To further characterize the coupling of nociceptive input to DH molecular changes, we conducted an RNA-Seq analysis of the DH of surgically incised animals after inactivation of TRPV1+ nociceptors with peripheral resiniferatoxin injection.

Materials and Methods

Animal Care

Experiments were approved by the Institutional Animal Care and Use Committee of the Clinical Center, National Institutes of Health (Bethesda, Maryland). Animals were cared for and tested in accordance with ethical guidelines established in the Guide for Care and Use of Laboratory Animals. Male Sprague Dawley rats (200 to 300 g) were housed in pairs with 12-h light-dark cycles, fed *ad libitum*, and were tested and monitored for behavior during the animal's light cycle. Animal cages were furnished with a plastic tunnel for enrichment. Male and female Sprague Dawley rats have been shown to have similar nocifensive behavior after surgical incision, and similar responses to approved analgesics postoperatively,[20] so we focused on male rats in this study. Future work will extend this work to female rats.

Plantar Surgical Incision and Perioperative Analgesia

Animals received 25 μl of vehicle or resiniferatoxin (8 ng/μl in 0.25% Tween-80, 2 mM ascorbic acid, and normal saline) through a 30-gauge needle to the plantar area 15 to 25 min before incision. An injection was made to subcutaneous and intramuscular tissues. Surgical incision on the plantar aspect of the rat hind paw was made as previously described.[21] Briefly, a 1-cm longitudinal incision was made through skin and fascia with a no. 15 scalpel blade, and the incision was extended into the underlying plantar flexor digitorum brevis muscle with a no. 11 scalpel blade. The wound was closed with two horizontal mattress sutures. While resiniferatoxin causes initial discharge of nociceptors expressing TRPV1 before the terminals degenerate, transmission of the pain signal can be blocked with local anesthetics.[14] To address this issue, some rats received 2% lidocaine (Fresenius Kabi, USA) either alone or 5 min before resiniferatoxin injection, and this was administered by the same route as vehicle and resiniferatoxin. The timing of the administration was chosen to approximate clinically relevant procedural use. Injection and surgery were conducted under isoflurane anesthesia. As the addition of lidocaine had no significant effect on thermal hyperalgesia or mechanical allodynia (Supplemental Digital Content 1, http://links.lww.com/ALN/B575, which shows the effect of lidocaine on postincisional nocifensive behavior), animals treated with lidocaine were pooled with those not treated with lidocaine for behavioral analysis.

Evoked Measurements of Nociception and Hyperalgesia

An infrared diode laser (LASS-10 M; Lasmed, USA) with an output wavelength of 980 nm was used to generate thermal stimuli as previously described.[10] Briefly, unrestrained rats were placed on a glass platform under a plastic enclosure. Thermal C-fiber–mediated behavioral responses were evoked using a continuous 5-mm–diameter beam at 1,000 mA, and the time until paw withdrawal was measured, or the assay was stopped at 25 s if no withdrawal occurred. At paw withdrawal, the surface temperature of the paw was inferred from a standard curve (Supplemental Digital Content 2A, http://links.lww.com/ALN/B576, which shows the relationship between continuous paw heating and paw surface temperature). Thermal A-δ fiber–mediated fast withdrawal reflexes were evoked using a 100-ms pulse of laser stimulation at 3,500, 4,500, or 5,000 mA with a 1.6-mm–diameter beam; approximate temperature changes from these stimuli are shown in Supplemental Digital Content 2B (http://links.lww.com/ALN/B576). Paw withdrawal to the 100 ms A-δ laser pulse was measured as a binary variable.

Submitted for publication May 22, 2017. Accepted for publication October 26, 2017. From the Department of Perioperative Medicine, Clinical Center, National Institutes of Health, Bethesda, Maryland (S.J.R., M.R.S., D.M.L., M.J.I., A.J.M.); and the Cleveland Clinic Lerner College of Medicine, Case Western Reserve University, Cleveland, Ohio (S.J.R.).

The small-diameter A-δ beam was targeted toward the distal, medial edge of the surgical incision. The interstimulus interval was 5 min with successive delivery of the 3 small-diameter A-δ stimulations first, followed by stimulation with the larger-diameter C-fiber beam. Calibrated Von Frey monofilaments were used to assess mechanical allodynia as described previously.[21] Briefly, animals were placed on an elevated wire mesh surface, and calibrated monofilaments of increasing strength were applied to the distal, medial edge of the surgical incision until consecutive withdrawals were observed or 60 g (the cutoff value) was reached. The cutoff value 60 g was recorded even if there was no withdrawal response with this force. To further assess allodynia to punctate mechanical stimuli, Von Frey filaments producing a bending force of 0.4 g, 1 g, 2 g, 4 g, or 6 g were applied near the distal edge of the wound for 1 to 3 s. Filaments were tested 10 times in order of increasing force, and the number of brisk paw withdrawals was counted.[22]

Spontaneous Nocifensive Behavior and Motoric Function

Spontaneous guarding of the injured paw was scored as previously described.[21] These measurements were made before other behavioral assessments and recorded using a Canon HF R700 camcorder (Japan). Briefly, rats were placed on an elevated wire mesh surface (7 × 7 mm) and video-recorded from below for 3 consecutive minutes while they were free to behave naturally. Animals were scored in 1-s bins based on the position of the paw according to a previously established rubric as follows.[23] If the area of the wound was blanched or distorted by the mesh, indicating the animal was using this foot for weight-bearing, a score of 0 was given; if the area of the wound was touching the mesh but without any blanching or distortion, indicating the animal was supporting some of its weight using this paw, a score of 1 was given; if the area of the wound was not in contact with the wire mesh, a score of 2 was given. The sum of the scores during the 3 min was used as a measure of postincision guarding. Motoric function was assessed using an accelerating rotarod (Model 7750, Ugo Basile, Italy) set to accelerate from 4 to 50 rpm over a period of 3 min. Rats were habituated over 4 consecutive days before data were collected. Latency to first fall was recorded.

Immunohistochemistry

Rats were anesthetized with ketamine and xylazine on postoperative day 5 and transcardially perfused with phosphate buffered saline followed by 4% paraformaldehyde. Lumbar spinal cord segments were dissected, postfixed in 4% paraformaldehyde overnight, and embedded in paraffin blocks from which 6-μm sections were cut. The slides were warmed at 60°C for 20 min, deparaffinized in xylenes three times for 5 min each, and hydrated in a decreasing ethanol gradient (100%, 95%, 70%, 30%, distilled H_2O) for approximately 1 min each. Sections were subjected to antigen retrieval performed using citrate buffer (10 mM citric acid, 0.05% Tween-20, pH 6.0) in a 1200-W microwave for 3 min at 100% power and 10 min at 30% power. Slides were washed in buffer containing 145 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 10 mM HEPES. Tissue sections were blocked by incubation in rabbit serum (VECTASTAIN Elite ABC-HRP Kit, peroxidase, and goat IgG, Vector Labs, USA) for 30 min. The slides were incubated in primary antibody (Iba1, 1:2,000, Abcam, USA) and diluted in antibody diluent (1% bovine serum albumin, 0.05% sodium azide, and 0.1% Tween-20 in phosphate buffered saline) for 1 h. The slides were washed in buffer for 5 min and incubated in biotinylated secondary antibody from the VECTASTAIN Kit for 30 min. The slides were washed again for 5 min in buffer and incubated in the Vector ABC Reagent for 30 min. The slides were washed for 5 min in buffer and developed with the ImmPACT DAB kit (Vector Labs, USA) until optimal color developed (7 min). The slides were rinsed in tap water and counterstained with hematoxylin (Sigma-Aldrich, USA). Scans of all slides were taken with an Aperio CS2 Scanner (Leica Biosystems, USA).

Tissue Preparation and RNA Purification

Rat DH tissue was dissected on postoperative day 2 and frozen at –80°C. Total RNA was extracted following the protocol from the RNeasy Lipid Tissue Mini Kit including the optional DNase digestion (Qiagen, USA). Briefly, tissue was homogenized in Qiazol (Qiagen) using the Fast Prep-24 Homogenizer (MP Biomedicals, USA). Chloroform was added, and the aqueous layer was extracted. An equal volume of 70% ethanol was added, and the RNA was bound to the spin column. The column was washed and then incubated with RNase-Free DNase (Qiagen) at room temperature for 15 min. The columns were washed again, and RNA was eluted off the column in RNase-free water. RNA integrity was evaluated using a 2,100 Bioanalyzer and the RNA 6,000 Nano Kit (Agilent Technologies, USA). All samples had RNA integrity score greater than or equal to 8.9.

Alignment and Quantification of RNA-Seq Count Data

Sequencing was performed at the National Institutes of Health Intramural Sequencing Center as described previously.[24] Briefly, messenger RNA libraries were constructed from 22 rat DH samples using 0.6 to 1 μg messenger RNA and Illumina TruSeq RNA Sample Prep Kits, version 2 (Illumina, USA). The resulting complementary DNA was fragmented using a Covaris E210 instrument. Library amplification was performed using 10 cycles to minimize overamplification. Unique barcode adapters were applied to each library. Libraries were pooled in equimolar ratio and sequenced together on a HiSeq 2,500 (Illumina) with version 4 flow cells and sequencing reagents. For each individual library, 35 to 45 million 125-base read pairs were generated. Data was processed using real-time analysis 1.18.61 or 1.18.64 and CASAVA 1.8.2 software tools (Illumina). Resulting Binary Alignment Map files were converted to fastc format using Picard tools and the mRNA and

Genome Integrative Cooperative (MAGIC) software suite.[25] Gene quantification was performed using a rat genomic target based on RefSeq annotations for the rn6 genome and the National Center for Biotechnology Information MAGIC pipeline. Quantification and normalization of gene expression is reported in significant fragments per kilobase of transcript per million mapped reads (sFPKM), a variant of fragments per kilobase of transcript per million mapped reads corrected to limit the influence of protocol biases.[25] Differentially expressed genes (DEGs) are identified by comparing the distributions of gene expression of each gene across two experimental groups of samples. Each gene is given a DEG score in the range 0 to 200, where 200 indicates a perfectly classifying gene between the two conditions examined. A threshold is chosen by controlling the false discovery rate (FDR) in a gene-by-gene fashion. To identify genes that are highly variable for a cause unrelated to the condition being studied, 80 random permutations are constructed orthogonal to the phenotype under study: in each permutation, the two random groups contain the same proportion of samples belonging to the two experimental groups.[26] The average "noise" score for each gene across these 80 permutations is measured and subtracted from the experimental DEG score for that gene. The average number of "noise" genes above a score is compared to the number of experimental "signal" genes above the same score to calculate the FDR. The threshold defining the significant DEG score is chosen to maintain the overall pairwise FDR less than 5% without exceeding an incremental FDR of 20% upon addition of potential differentially expressed genes (Supplemental Digital Content 3, http://links.lww.com/ALN/B577, which shows the relationship between FDR and differential score threshold). The MAGIC software, documentation, and targets are available at ftp://ftp.ncbi.nlm.nih.gov/repository/acedb/Software/Magic (accessed September 25, 2017). DEGs were correlated with a public database of central nervous system cell-type specific gene expression.[27] Cell-type specific marker genes were defined as those with threefold increased expression in one cell type *versus* any other cell type. Raw data are available upon request and downloadable from the sequence read archive database under project number PRJNA412076.

Weighted Gene Coexpression Network Analysis and Functional Analysis

Weighted Gene Co-expression Network Analysis (WGCNA) allows unbiased detection of networks of gene modules. Briefly, a signed Pearson correlation matrix was constructed among all pairs of genes with variance greater than 1 across control DH samples and samples of DH ipsilateral to the incision. The correlation matrix was increased to the power $\beta = 18$ to fit the assumption of scale-free topology and to calculate an adjacency matrix, and this was transformed to a topological overlap matrix (TOM). Genes with similar expression pattern were clustered based on hierarchical clustering of the matrix 1-TOM. Resulting modules were merged if the first principal components of the modules had a Pearson correlation exceeding 0.80, and modules were considered significantly correlated with surgery if the *P* value was less than 0.05 after the Holm-Sidak procedure for multiple tests. The analysis was performed using custom R scripts. Gene ontology (GO) enrichment analysis was performed using g:Profiler on the genes in the gene module correlating with surgery focusing on terms in the biologic process domain.[28] GO terms were considered significantly enriched ($P < 0.05$) after Bonferroni correction.

Statistical Analysis of Behavioral Assays

Animals (n = 51) were randomized to treatments, and the experimenter was blinded to treatment condition for all behavioral assays. All statistical tests were two-sided, and a *P* value of less than 0.05 was considered statistically significant. No data were excluded or lost. Some cohorts of animals were euthanized for biochemical analyses before postoperative day 10. Sample sizes for each assay, at each time point, and for each experimental condition can be found in Supplemental Digital Content 4 (http://links.lww.com/ALN/B578). Sample sizes were chosen based on our group's previous experience working with resiniferatoxin in rats. Exact *P* values for behavioral assays are reported in the text or can be found in Supplemental Digital Content 4 (http://links.lww.com/ALN/B578). To compare baseline and postsurgical continuous outcomes, the Kruskal-Wallis test with the Dunn multiple comparison procedure was used. In the setting of planned experimental loss of animals for biochemical analyses, we did not account for repeated measures on individual animals when comparing postsurgical measurements to baseline values. Without planned experimental loss of animals, continuous outcomes were compared with two-way repeated-measures ANOVA with Holm-Sidak step-down method adjustments for multiple comparisons. To compare treated and untreated animals after plantar surgery, repeated Mann–Whitney U tests were used at each time point with Holm-Sidak step-down method adjustments for multiple comparisons. Results are reported as median with interquartile range. The Fisher exact test was repeated at each time point with Holm-Sidak step-down method adjustments for multiple comparisons to compare treated and untreated animals for binary response variables. Analyses were performed with Prism Statistical Software (GraphPad, USA).

Results

Resiniferatoxin Blocks Thermal Hyperalgesia after Surgical Incision

Resiniferatoxin was administered in a clinically relevant timeline in the perioperative period (fig. 1A). Plantar incision caused thermal hyperalgesia to continuous heat stimulation (fig. 1B) as indicated by a decrease in median withdrawal latency to 2.6 s (2.25 to 3.25 s) at 4 h after surgery (76% decrease from a baseline of 10.85 s with interquartile range

9.58 to 13.45 s). Hyperalgesia remained prominent until postoperative day (POD) 3, when latency was 5.75 s (47% of baseline, $P < 0.05$, Kruskal-Wallis test with the Dunn multiple comparisons test; see Supplemental Digital Content 4 at http://links.lww.com/ALN/B578 for exact P values for comparison of each postoperative day). This effect was reversed in the resiniferatoxin treated group, and resiniferatoxin-treated animals had withdrawal latencies that were 2.3- to 9.6-fold longer compared to vehicle-treated animals through POD4 ($P < 0.05$, repeated Mann–Whitney U tests with Holm-Sidak adjustment). After resiniferatoxin treatment, animals did not withdraw their paws on average until temperatures exceeded 47°C through POD6 (see heating curve in Supplemental Digital Content 2A, http://links.lww.com/ALN/B576).

Plantar incision caused increased withdrawal responsiveness at the three different thermal A-δ stimulus intensities after surgical incision. Even with the lowest thermal stimulus (see heating curve in Supplemental Digital Content 2B, http://links.lww.com/ALN/B576), plantar incision caused increased withdrawal responsiveness (hyperalgesia) through POD1 to the brief thermal pulses (thermal A-δ mediated rapid withdrawal reflex, fig. 1C), with 77% of vehicle-treated animals withdrawing compared to 0% of resiniferatoxin-treated animals ($P = 0.0009$, Fisher exact test with Holm-Sidak correction for multiple comparisons). Responsiveness to the medium- and high-intensity (fig. 1, D and E) thermal pulses was significantly blocked in the resiniferatoxin-treated group after incision through POD4 and POD10, respectively ($P < 0.05$, Fisher exact test with Holm-Sidak correction

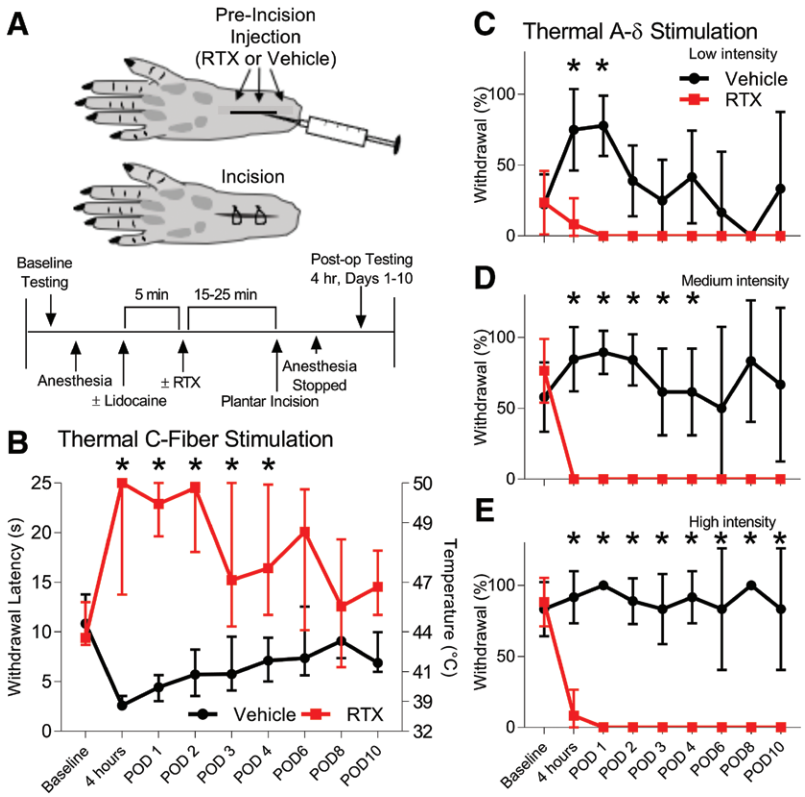

Fig. 1. Postincisional thermal hyperalgesia is attenuated after peripheral resiniferatoxin injection. (*A*) *Top*, Diagram for the location of peripheral injection and plantar surgical incision. *Bottom*, Experimental timeline for behavioral testing with sequence of anesthesia induction, peripheral injections of analgesics, and plantar incision. (*B*) Plantar incision led to decreased withdrawal latency to continuous thermal stimulation associated with C-fiber activation, which was attenuated by resiniferatoxin through POD4 (*$P < 0.05$, repeated Mann–Whitney U tests with Holm-Sidak adjustment; data are plotted as median with IQR). (*C*) Hyperalgesia to low-intensity 100-ms thermal pulse associated with A-δ fiber stimulation (maximum temperature, ~64°C) was most pronounced through POD1 and attenuated by resiniferatoxin during this time (*$P < 0.05$, repeated Fisher exact tests with Holm-Sidak adjustment; *error bars* are 95% CIs). (*D*) Hyperalgesia to moderate-intensity thermal stimulation (maximum temperature, ~105°C) was evident for several days after surgical incision, and resiniferatoxin treatment significantly attenuated the withdrawal response through POD4 (*$P < 0.05$, repeated Fisher exact tests with Holm-Sidak adjustment). (*E*) Most vehicle-treated animals withdrew from the high-intensity thermal pulse (maximum temperature, ~124°C), but resiniferatoxin ablated this response through POD10 (*$P < 0.05$, repeated Mann–Whitney U tests with Holm-Sidak adjustment). Thermal A-δ mediated withdrawal reflexes take 3 weeks or longer to return to baseline, depending on the intensity of the stimulus.[38] See Supplemental Digital Content 2 (http://links.lww.com/ALN/B576) for the relationship between laser intensity and paw surface temperature. IQR = interquartile range; POD = postoperative day; RTX = resiniferatoxin.

for multiple comparisons). As TRPV1 mediates the sensation of noxious heat, these data signify successful ablation of TRPV1$^+$ terminals by peripheral resiniferatoxin, as well as the peripheral sensitization of TRPV1$^+$ fibers after incision.

Resiniferatoxin Decreases Nonthermal Nocifensive Behaviors

Resiniferatoxin attenuated nonthermal nocifensive behaviors in the postoperative period (fig. 2). In vehicle-treated animals, plantar incision led to a reduced Von Frey mechanical withdrawal threshold (fig. 2A) at 4 h postoperatively, and this mechanical hyperalgesia remained through POD4 (98% reduction from baseline to 88% reduction, $P < 0.05$, Kruskal-Wallis test with the Dunn multiple comparisons test). Resiniferatoxin treatment attenuated the hyperalgesic response through POD2 with resiniferatoxin-treated animals having at least a sevenfold higher mechanical withdrawal threshold during this time compared to vehicle-treated animals ($P < 0.05$, repeated Mann–Whitney U tests with Holm-Sidak adjustment). Mechanical allodynia was assessed with a second complementary test using repeated application of low-intensity mechanical stimuli, and peripheral resiniferatoxin was found to decrease the median number of paw withdrawals by 50% for stimuli with bending forces of 2 to 6 g through POD2 (fig. 3). When peripheral resiniferatoxin was given 1 day after incision, after mechanical allodynia had developed, paw withdrawal response was attenuated relative to vehicle-treated animals ($P < 0.05$ for filaments with bending forces greater than or equal to 2 g, two-way repeated-measures ANOVA with *post hoc* comparison to vehicle control with Holm-Sidak adjustment). Nonevoked measures of pain were also attenuated by perioperative resiniferatoxin treatment. Vehicle-treated animals exhibited paw guarding behavior through POD2 (fig. 2B), whereas resiniferatoxin-treated animals demonstrated a 90% or greater reduction in guarding score compared to vehicle-treated animals during this time ($P < 0.05$, repeated Mann–Whitney U tests with Holm-Sidak adjustment). Treatment with resiniferatoxin had no effect on motoric function (fig. 2C), nor was there any effect of resiniferatoxin in response to pinprick (see Supplemental Digital Content 5, http://links.lww.com/ALN/B579, which is a figure of animal response to pinprick), demonstrating that resiniferatoxin peripheral ablation did not indiscriminately eliminate all pain-sensing modalities. There was no evidence of autotomy[29,30] such as paw gnawing.

Plantar Incision with or without Preemptive Resiniferatoxin Leads to Ipsilateral Gene Changes in the Dorsal Spinal Cord

Plantar incision induced expression of 70 genes compared to a separate group of control rats; no genes exhibited decreased expression (fig. 4, A and B). Using a within-animal comparisons, changes were specific to the DH ipsilateral to surgical incision; ipsilateral DH had 92 genes with increased expression compared to the contralateral DH (fig. 4C). Despite the evident level of analgesia achieved during our behavioral assays, in animals pretreated with resiniferatoxin, we still observed an induction of 37 genes in the DH compared to control (fig. 4D), and no genes were significantly different in the DH of incised animals with and without preemptive resiniferatoxin (fig. 4E). The transcriptomic changes in the DH after surgical incision with and without resiniferatoxin were highly similar by covariance analysis, indicating similar expression levels of almost all genes in these two data sets (Supplemental Digital Content 6, http://links.lww.com/ALN/B580). While fewer genes were significantly induced after incision with resiniferatoxin (37 genes) compared to without resiniferatoxin (70 genes), this is largely because the incision in the resiniferatoxin group had greater variability in

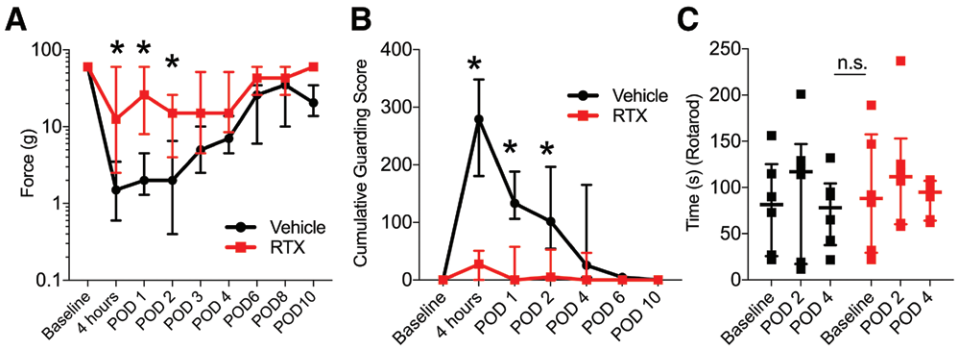

Fig. 2. Postincisional mechanical allodynia, spontaneous pain (guarding score), and motoric function. Data are plotted as median with IQR. (*A*) Withdrawal threshold to mechanical stimuli is decreased after surgery, but the allodynia is significantly attenuated after resiniferatoxin (RTX) treatment through POD2 (*$P < 0.05$, repeated Mann–Whitney U tests with Holm-Sidak adjustment). (*B*) Paw guarding score, a measure of spontaneous nocifensive behavior, was increased from baseline after plantar incision; resiniferatoxin treatment attenuated guarding behavior through POD2 (*$P < 0.05$, repeated Mann–Whitney U tests with Holm-Sidak adjustment). (*C*) Treatment with resiniferatoxin did not change the duration of time the rats spent on the rotarod, a measure of motoric function ($P > 0.50$, repeated Mann–Whitney U tests). IQR = interquartile range; n.s. = not significant; POD = postoperative day.

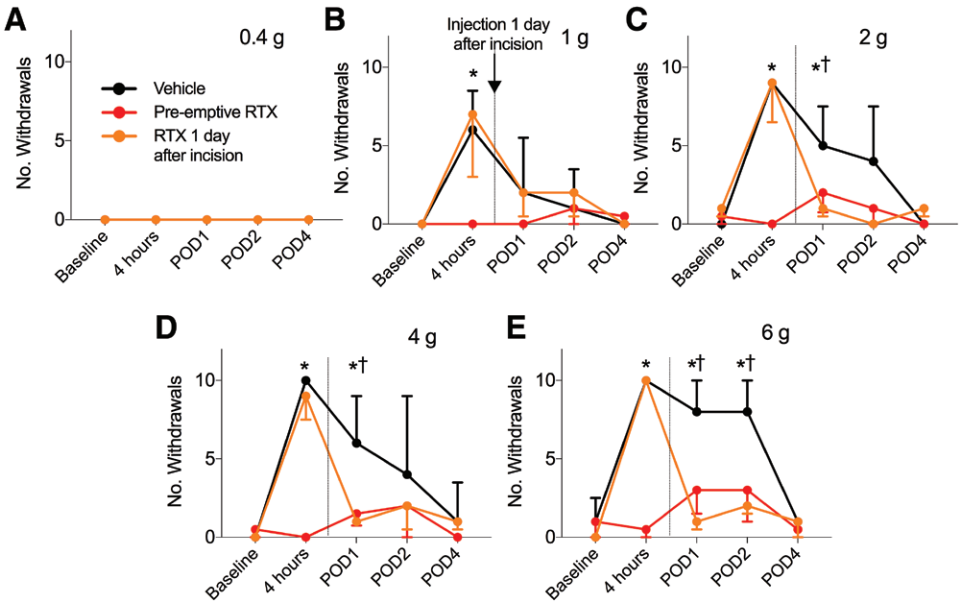

Fig. 3. Resiniferatoxin (RTX) attenuates postsurgical mechanical allodynia when given preemptively (*red line*) or after the establishment of hypersensitivity (*orange line*). Data are plotted as median with IQR. (*A*) A range of filaments were assessed including a 0.4-g filament, which did not induce a behavioral response. (*B–E*) Preemptive resiniferatoxin (n = 6, *red line*) attenuated behavioral responses to 1-g, 2-g, 4-g, and 6-g filaments, indicating a reduction in postincisional mechanical allodynia with resiniferatoxin treatment. (*$P < 0.05$, two-way repeated measures ANOVA with *post hoc* comparison to vehicle control with Holm-Sidak adjustment). Resiniferatoxin was given 1 day after surgical incision (n = 6, *orange line*) with testing at least 2 h after intraplantar resiniferatoxin injection. Animals demonstrated attenuation of mechanical allodynia compared to vehicle control (†$P < 0.05$, two-way repeated-measures ANOVA with *post hoc* comparison to vehicle control with Holm-Sidak adjustment). Vehicle-treated control n = 5. IQR = interquartile range; POD = postoperative day.

gene expression, and with our stringent requirement for controlling the FDR, fewer genes had the magnitude of induction needed to overcome this variability and reach statistical significance (see Supplemental Digital Content 3, http://links.lww.com/ALN/B577, which demonstrates the significance threshold to maintain the FDR less than 5% in different experimental comparisons).

Because many genes are changed by surgical incision, but not all surpass the threshold for statistical significance when corrections for multiple comparisons are made, we further investigated subthreshold changes of related genes. WGCNA allows for unbiased detection of gene networks; comparing how these modules are affected by surgical incision allows us to identify significant gene networks that are composed of correlated genes, some of which underwent subthreshold elevation. Analysis of the 6,029 genes with variance greater than 1 across samples detected 27 distinct gene modules (fig. 5A). One module (blue module in fig. 5A) was significantly correlated with surgery ($P < 0.001$ after Holm-Sidak adjustment, fig. 5B) and contained 394 genes (see Supplemental Digital Content 7, http://links.lww.com/ALN/B581, for gene list with sFPKM values). Most genes changing with surgery did not change in the contralateral DH. Furthermore, peripheral resiniferatoxin pretreatment failed to significantly attenuate the induction of many genes (fig. 5C). GO term analysis of the gene module correlating with surgery identified gene enrichment for biologic processes relating to mitotic and apoptotic processes as well as immune cell activation, migration, and differentiation (fig. 5D; a full list of GO terms can be found in Supplemental Digital Content 8, http://links.lww.com/ALN/B582). The transcription levels of select genes in the DH for each experimental condition are shown in table 1, highlighting genes involved in the complement and chemokine signaling pathways, opioid pathways, and lipid pathways as well as select G-protein–coupled receptors (GPCRs) and select glial marker genes. The full list of genes with sFPKM values can be found in Supplemental Digital Content 9 (http://links.lww.com/ALN/B583), and the full list of genes with counts of aligned reads can be found in Supplemental Digital Content 10 (http://links.lww.com/ALN/B584).

Dynorphin Induction and Microglial Activation

Plantar incision led to a greater than 118% increase in expression of the prodynorphin gene (*Pdyn*) in the ipsilateral DH, but not in the contralateral DH relative to control animals (fig. 6A). The expression of *Pdyn* was not significantly different between the resiniferatoxin-treated and control animals (with only 49.5% increase in expression after incision with resiniferatoxin pretreatment compared to baseline). In contrast, many genes specific to microglia such as *Itgal* were increased after surgical incision irrespective of resiniferatoxin pretreatment (fig. 6, B–D), and known microglial activation markers *Itgam* and *Aif1*, which encode CD11b and Iba1,

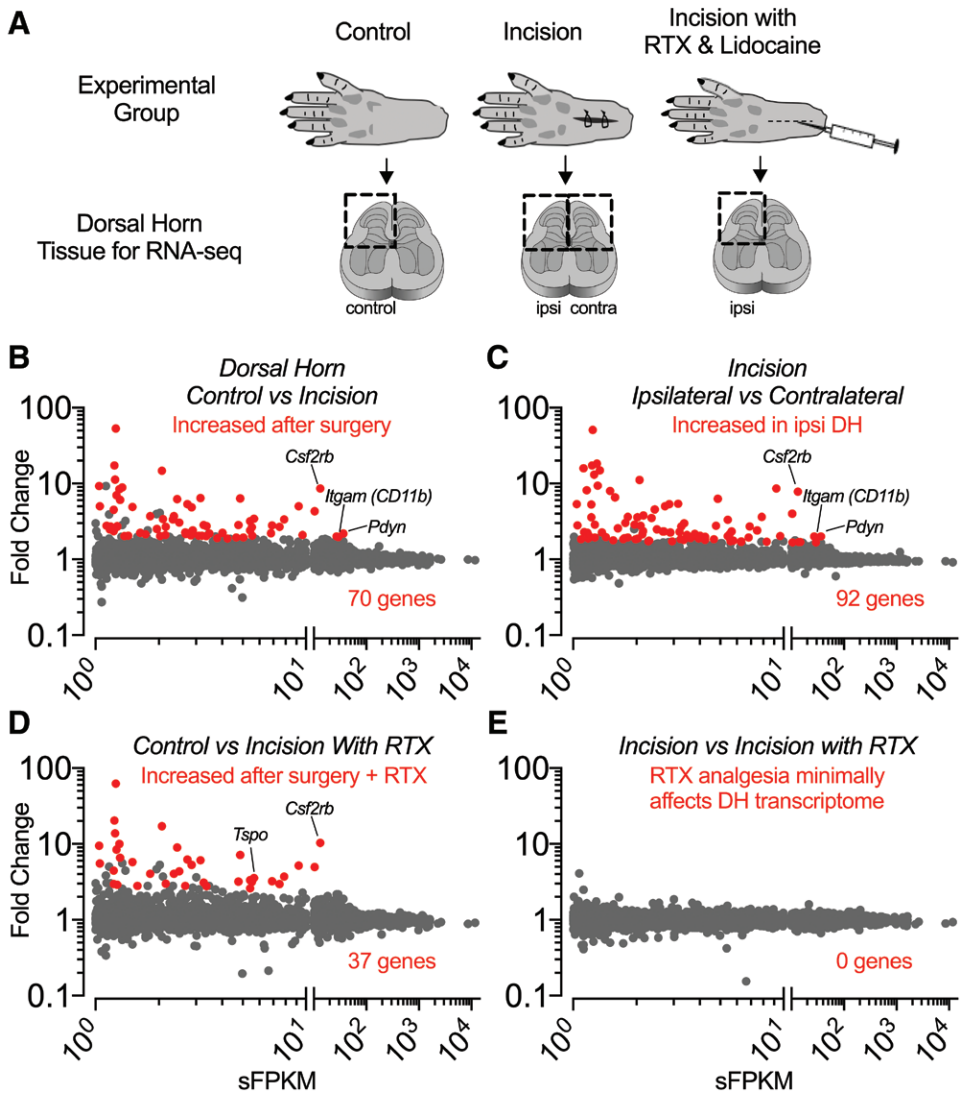

Fig. 4. Transcriptional changes in the DH on postoperative day 2 after plantar incision. (*A*) Schematic of experimental groups for RNA sequencing. Tissue was harvested on postoperative day 2. (*B*) Surgical incision increased gene expression in the DH relative to control (70 genes), and (*C*) similar changes were observed in the side of the DH ipsilateral to the incision relative to the contralateral side. (*D*) Surgically incised animals with preemptive resiniferatoxin (RTX) analgesia also had increased gene expression in the DH compared to controls (37 genes). (*E*) There were no significantly differential genes between the surgically incised group with or without intraplantar resiniferatoxin treatment. All genes are plotted (*gray dots*) with significantly differentially expressed genes in *red*. Contra = contralateral; DH = dorsal horn; Ipsi = ipsilateral; RNA-Seq = RNA sequencing; sFPKM = significant fragments per kilobase of transcript per million mapped reads.

were also increased after surgery (fig. 6, E and F). The transcriptomic signature of microglia activation is nearly identical in the DH of animals after surgical incision whether or not they received preemptive resiniferatoxin. That is, there are no differences in expression of microglial-specific genes or other markers of microglial subtypes such as *Tmem119* or *Sall1*[27,31,32] between the two conditions (Supplemental Digital Content 11, A–G, http://links.lww.com/ALN/B585). Moreover, genes that are specific for monocytes relative to microglia such as *Trem1* and *Trem3*[33,34] remain negligible in expression after injury, suggesting a low contribution from myeloid cells invading the spinal cord in this model (Supplemental Digital Content 11, C and D, http://links.lww.com/ALN/B585).

We confirmed by immunohistochemistry that Iba1 was increased in DH lamina I and II ipsilateral to surgical incision compared to contralateral; pretreatment with resiniferatoxin had no effect on the number of Iba1 immunoreactive microglial cells (fig. 6, G and H). The immunohistochemical changes were observed at POD5, indicating that glial activation persists even as hyperalgesia is subsiding. Resiniferatoxin without surgical incision also caused a modest nonsignificant increase in the number of Iba1 immunoreactive microglial cells in the ipsilateral dorsal spinal cord compared

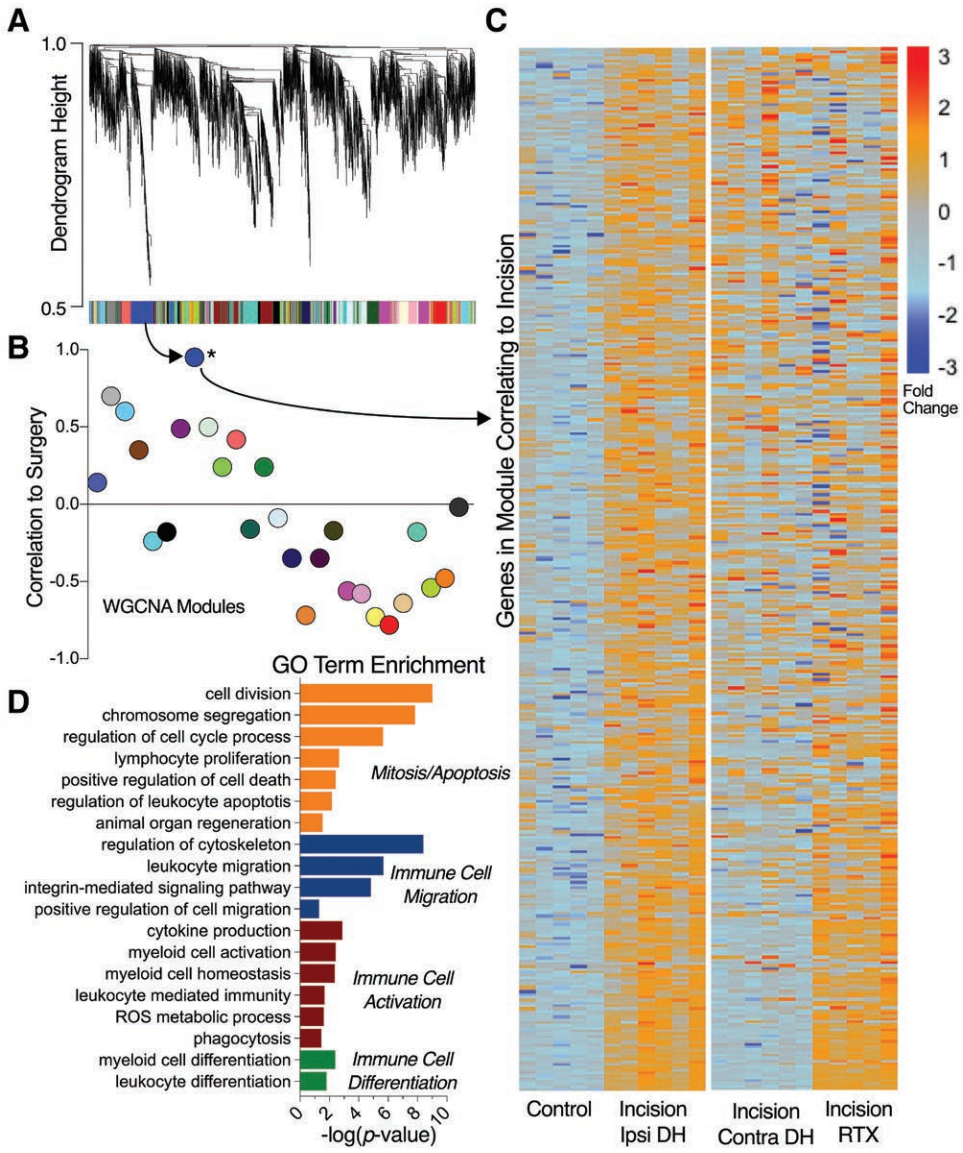

Fig. 5. (*A*) Weighted Gene Co-expression Network Analysis (WGCNA) was used to identify gene modules in control and surgically treated animals; genes are shown as terminal branches of the dendrogram, and groups of genes that are closely correlated are represented as large downward deflections in the graph. Distinct gene clusters (27 clusters) are marked with unique colors beneath the cluster dendrogram. (*B*) Each module is plotted as a *colored circle*. The *blue* gene module (394 genes) was the only gene module significantly correlated with surgery ($P < 0.001$ after Holm-Sidak adjustment). (*C*) The genes in the module are represented in a heat map that shows increased expression after surgery in the ipsilateral DH. Graphical representation for the expression of these genes in the contralateral DH and after preemptive resiniferatoxin analgesia are shown for comparison. Each column is an individual animal, and each row of the heat map corresponds to a gene; expression values (sFPKM) are normalized across each row. (*D*) Gene ontology (GO) analysis for the gene module that changes with plantar incision shows that predominant changes in the DH relate to mitosis signatures, immune cell migration, immune cell activation, and immune cell differentiation ($P < 0.05$ after Bonferroni correction; nonspecific terms not shown in figure; see Supplemental Digital Content 7, http://links.lww.com/ALN/B581, for full list of GO terms). Contra = contralateral; DH = dorsal horn; Ipsi = ipsilateral; ROS, reactive oxygen species; RTX = resiniferatoxin; sFPKM = significant fragments per kilobase of transcript per million mapped reads.

to the contralateral side (10.75 ± 3.48 *vs.* 8.25 ± 2.33, $P = 0.17$, paired *t* test, n = 4; Supplemental Digital Content 12, http://links.lww.com/ALN/B586). Iba1 immunoreactive microglial cells were located in the medial DH, consistent with innervation from the tibial nerve at the site of injury.

Discussion

Resiniferatoxin was efficacious in decreasing multiple measures of postoperative pain in rats. Resiniferatoxin binding causes prolonged TRPV1 channel opening, produces a large intracellular calcium overload, and rapidly leads to nerve terminal silencing.[15,16] Because of the long-term nature of nerve

Table 1. Gene Changes after Surgical Incision and with Preemptive Resiniferatoxin-based Analgesia

Gene	Protein Product	Control (sFPKM)	Ipsi (sFPKM)	Fold Change (Ipsi/Control)	Contra (sFPKM)	RTX (sFPKM)	Sig	DEG Score
	Opioid pathways							
Penk	Proenkephalin	145.8	165.4	1.1	148.3	160.9	n.s.	—
Oprl1	Nociceptin/orphanin FQ peptide receptor	39.5	38.5	1.0	37.9	36.7	n.s.	—
Pdyn	Prodynorphin	16.8	36.7	2.2	18.2	24.6	*	95.5
Pnoc	Prepronociceptin	13.5	13.1	1.0	13.1	14.2	n.s.	—
Oprk1	κ opioid receptor	5.8	5.9	1.0	5.9	6.0	n.s.	—
Oprm1	μ opioid receptor	6.0	6.5	1.1	6.1	7.3	n.s.	—
Oprd1	δ opioid receptor	4.9	4.7	0.9	4.7	4.9	n.s.	—
	Chemokine receptors							
Cx3cr1	Fractalkine receptor	40.1	53.0	1.3	41.3	53.4	#	—
Ccr5	C-C chemokine receptor type 5	7.9	11.8	1.5	8.4	10.4	#	—
Cmklr1	Chemokine like receptor 1/Chemerin Rec 23	2.0	3.1	1.6	2.4	2.6	#	—
	Complement system							
C3	Complement component C3	39.6	53.5	1.4	37.5	52.9	#	—
C1qa	A-chain polypeptide of C1q	32.0	47.2	1.5	33.4	47.7	#	—
C4a	Complement component 4a	13.1	21.9	1.7	15.8	23.5	*	78.5
C1qb	B-chain polypeptide of C1q	19.2	33.2	1.7	20.1	36.3	*	102.7
Cfh	Complement factor H	16.4	21.5	1.3	18.3	18.1	#	—
C1qc	C-chain polypeptide of C1q	8.2	15.0	1.8	8.9	14.5	*	99.8
Cfb	Complement factor B	5.5	9.2	1.7	7.9	7.7	#	—
	GPCRs							
Gpr84	G protein–coupled receptor 84	2.1	5.1	2.4	2.5	5.4	*	142.9
Niacr1	Hydroxycarboxylic acid receptor 2 (Hcar2)	2.2	4.0	1.8	2.1	3.8	*	109.6
Gpr31	G protein–coupled receptor 31	1.55	2.22	1.4	1.29	2.27	*	69.9
C3ar1	C3a anaphylatoxin chemotactic receptor	1.39	2.43	1.7	1.52	2.49	*	96.6
Gpr183	G protein–coupled receptor 183	1.2	2.5	2.1	1.5	3.1	*	117.9
P2ry6	P2Y purinoceptor 6	1.07	1.89	1.8	1.09	2.28	*	113.0
C5ar1	C5a anaphylatoxin chemotactic receptor	0.95	1.65	1.7	1.03	1.45	*	79.2
	Lipid pathways							
Pld4	Phospholipase D4	17.3	28.4	1.6	18.2	29.5	*	88.1
Pla2g4a	Cytosolic phospholipase A2	4.9	7.1	1.5	5.2	6.8	#	—
Tbxas1	Thromboxane A synthase 1	1.26	2.29	1.8	1.51	2.23	*	85.8
Alox5	Arachidonate 5-lipoxygenase	0.69	1.18	1.7	0.8	1.15	*	75.3
	Kinases							
Lyn	Tyrosine-protein kinase Lyn	7.1	10.7	1.5	7.3	10.3	*	70.5
Hck	Hematopoietic cell kinase	2.2	4.2	1.9	2.1	4.2	*	124.5
	Glial-related							
Gfap	Glial fibrillary acidic protein	1023.1	1147.8	1.1	918.6	1168.8	n.s.	—
Itgam	Integrin alpha M (CD11b)	12.5	25.0	2.0	12.5	27.2	*	131.1
Aif1	Allograft inflammatory factor (Iba1)	17.7	25.2	1.4	16.6	24.4	*	66.8
	Ion channels							
Clic1	Chloride intracellular channel protein 1	7.7	11.7	1.5	7.9	12.4	*	77.9
	Other potentially pain-related							
Csf2rb	Cytokine receptor common subunit beta	1.3	11.2	8.6	1.4	13.4	*	199.8
Tuba1c	Tubulin alpha-1C chain	2.1	9.1	4.3	2.3	10.5	*	187.0
Ptpn6	Tyr-protein phosphatase nonreceptor type 6	4.0	7.4	1.8	4.3	7.9	*	106.3
Casp1	Caspase 1	1.3	2.9	2.3	1.8	2.6	*	98.7

Mean gene expression (sFPKM) is expressed for the four experimental groups, and fold change is shown for the ratio of expression from control DH tissue to DH tissue from surgically manipulated animals. In order to increase power, experimental groups were grouped as follows for determination of statistical significance: (1) control DH with DH contralateral to surgical incision and (2) DH ipsilateral to surgical incision with and without resiniferatoxin. The threshold defining significant differentially expressed genes for the MAGIC differential gene analysis was set to a DEG score of 65 to maintain the overall false discovery rate less than 5% without exceeding an incremental false discovery rate of 20% upon addition of potential differentially expressed genes. See also Supplemental Digital Content 3 (http://links.lww.com/ALN/B577).

*Score above threshold by MAGIC Differential Gene analysis. †If not above threshold by MAGIC, member gene of the module correlating with surgery from WGCNA analysis ($P < 0.001$ for gene module correlation with surgery after Holm-Sidak adjustment).

DEG = differentially expressed gene; GPCR = G-protein–coupled receptor; n.s. = not significant; sFPKM = significant fragments per kilobase of transcript per million mapped reads; Sig = significant; WGCNA = Weighted Gene Co-expression Network Analysis.

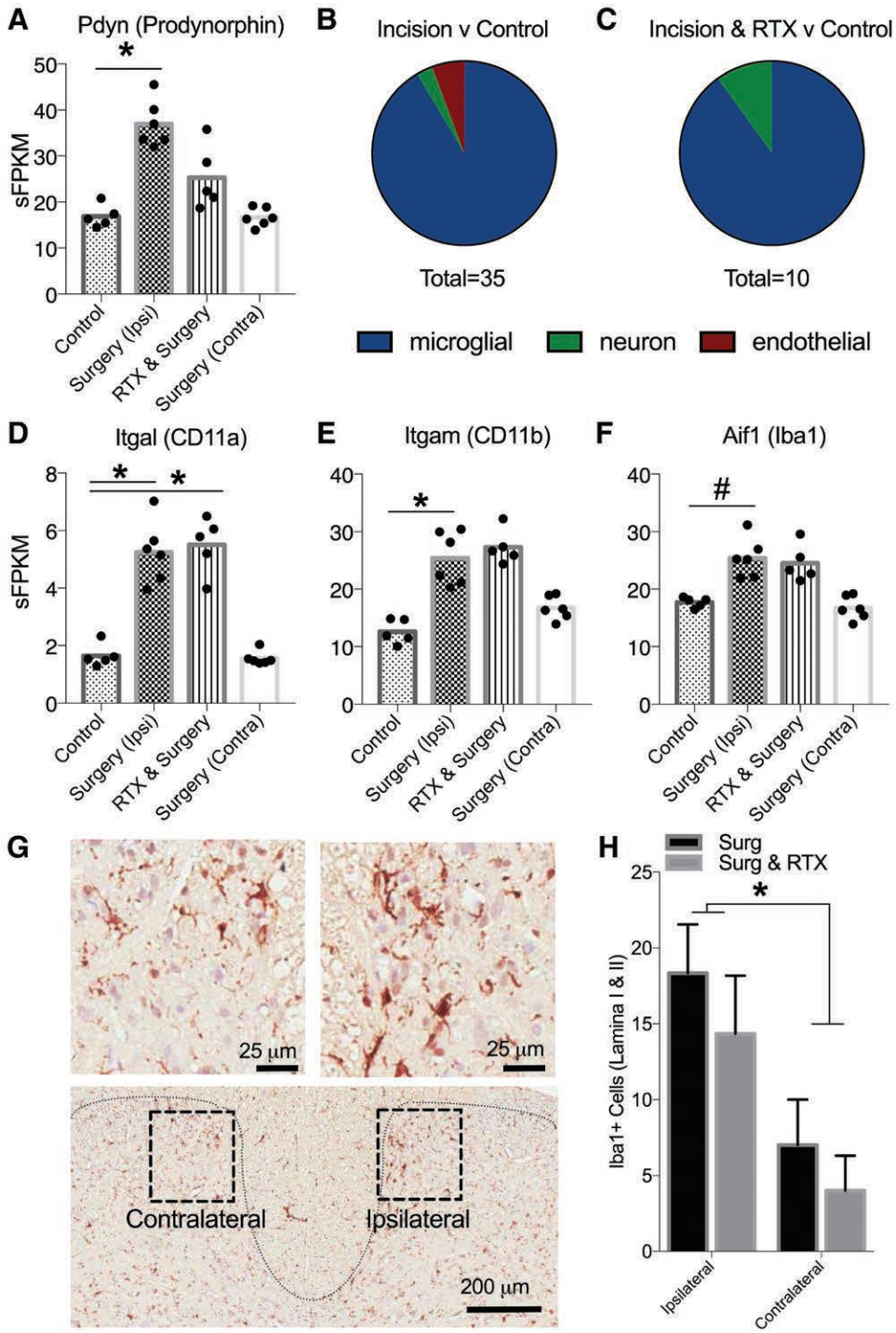

Fig. 6. Changes in prodynorphin (*Pdyn*) and microglial markers. (*A*) Of opioid receptors and peptides, only the transcript for *Pdyn*, which codes for prodynorphin, is upregulated. (*B*) Differentially expressed genes were correlated with a public database[27] of cell-specific gene expression. Tissue-specific expression marker genes were defined as threefold increased expression over other cell types. (*C*) Incision mainly upregulated genes that are markers of microglia even after perioperative resiniferatoxin administration. (*D*) Transcripts for microglial specific marker *Itgal* are increased after surgical incision with or without resiniferatoxin administration. Microglial activation markers *Itgam* (*E*) and *Aif1* (*F*) are increased after surgical incision. (*G*) Dorsal horn immunohistochemical staining for Iba1 confirms microglial activation on POD5 (outline of spinal gray matter shown by *dotted line*). (*H*) The ipsilateral dorsal spinal cord contained significantly more Iba1$^+$ cells in lamina I and II relative to the contralateral side ($P = 0.015$ for ipsi *vs.* contra, two-way repeated-measures ANOVA, n = 3 for both treatment groups, data plotted as mean with SD). For (*A, D–F*), *score above threshold by MAGIC Differential Gene analysis; #member gene of the module correlating with surgery from WGCNA analysis. Contra = contralateral; Ipsi = ipsilateral; POD = postoperative day; RTX = resiniferatoxin; sFPKM = significant fragments per kilobase of transcript per million mapped reads; Surg = surgery; WGCNA = Weighted Gene Co-expression Network Analysis.

terminal inactivation, analgesia with resiniferatoxin outlasts the period of greatest postincisional thermal hyperalgesia (fig. 1), mechanical allodynia (figs. 2A and 3), and spontaneous nonevoked pain (fig. 2B). To realistically model perioperative usage, resiniferatoxin was administered peripherally just minutes before surgical incision. Previously, perineurally applied resiniferatoxin was shown to be an efficacious analgesic for surgical incision and other pain models.[35,36] The current study uses a less invasive and more timely peripheral administration protocol better suited for a clinical setting. Tissue infiltration with local anesthetics, with the possible exception of liposomal bupivacaine,[37] provides a short duration of analgesia, and while many regional nerve blocks provide long-lasting analgesia, they impair motor function and may require placement of a perineural catheter.

In comparison to local anesthetics, nerve terminal inactivation with resiniferatoxin is sensory-modality selective, such that protective withdrawal reflexes to noxious mechanical stimuli like a pinprick are preserved (Supplemental Digital Content 5, http://links.lww.com/ALN/B579). The analgesic action of resiniferatoxin, although long-lasting, is temporary, and restoration of impaired pain sensation returns as TRPV1$^+$ nerve terminals regrow and reinnervate the tissue. Thermal C-fiber–mediated behavioral responses return to baseline before 3 weeks while thermal A-δ–mediated withdrawal reflexes take 3 weeks or longer, depending on the intensity of the stimulus.[38] The efficacy of peripheral resiniferatoxin, along with the sparing of noxious mechanical sensation and motor coordination, indicates a high potential for the perioperative setting.

Because resiniferatoxin inactivates the entire peripheral TRPV1$^+$ nerve terminal, analgesia is achieved beyond that which could be achieved by blocking only TRPV1 signal transduction. For example, TRPV1 antagonists[39–41] and *Trpv1*$^{-/-}$ mice[11,18] continue to exhibit mechanical allodynia after incision, whereas full inactivation of the TRPV1$^+$ terminal produced robust attenuation as assessed by the Von Frey test (figs. 2A and 3). Capsaicin has also been used to impair TRPV1$^+$ nociceptor signaling, but in comparison, resiniferatoxin is more potent[15] and more rapidly leads to attenuation of paw guarding, thermal hyperalgesia, and mechanical allodynia.[42–44] The mechanism by which TRPV1$^+$ nerve terminal inactivation attenuates mechanical allodynia after surgical incision is not fully known. Inactivation of TRPV1$^+$ terminals with capsaicin diminishes mechanical allodynia with surgical incision when given before or after incisional hyperalgesia has developed,[44] suggesting that TRPV1$^+$ fibers mediate part of the mechanical sensitivity after surgical incision. However, capsaicin attenuation of mechanical allodynia is not seen in all inflammatory conditions.[45] Peripheral resiniferatoxin, given 1 day after surgical incision, also attenuates mechanical allodynia, suggesting TRPV1$^+$ nerve terminals are involved in the maintenance of postsurgical mechanical hypersensitivity (fig. 3).

To better elucidate the molecular pathophysiology of postsurgical pain, we studied transcriptomic changes in the DH after incision. Gene changes in the DH after surgical incision were specific to the DH tissue ipsilateral to the site of surgical manipulation (fig. 4), and all genes were induced (70 genes exhibited increased expression *vs.* 0 genes decreased compared to control). Because the changes were unilateral, they are unlikely to be due to systemic factors induced by surgical manipulation. We discovered that many genes induced in the DH by surgical incision are related to immune cell signaling. We cross-validated this finding using WGCNA, identifying gene modules related to immune cell signaling induced after surgical incision (fig. 4D). These observations are consistent with microglial activation (fig. 6, B and C), and genes showing increased expression include microglial markers coding for Iba1 and Cd11b (fig. 6, E and F), among many others (Supplemental Digital Content 11, http://links.lww.com/ALN/B585). There has been extensive work on the role of microglial activation in different pain models (see reviews[46,47]), and activation of p38 MAPK in spinal microglia has been shown to contribute to postsurgical mechanical allodynia.[48] Transcripts for chemokines such as fractalkine as well as many transcripts involved in the complement system were induced after surgical incision (table 1); these potentially play a role in hypersensitivity, especially after neuropathic injury.[49–51]

This is the first report using RNA-Seq to quantitatively and comprehensively address gene changes in the DH after peripheral injury, and this database can serve as a resource for further investigations of incision-induced central changes. Select transcriptomic changes are categorized in table 1. As there were no differentially expressed genes within control DH samples compared to contralateral DH samples, these experimental treatments were grouped together to identify genes for table 1. Likewise, ipsilateral DH samples with and without preoperative resiniferatoxin were also grouped together. Combining the groups allowed more statistical power to detect changes in the postsurgical DH. The purinergic receptor *P2ry6* and the GPCR *Gpr84* are induced in the DH after surgical incision. Previous work shows these genes participate in nociception after nerve injury.[52,53] The GPCR *Niacr1*, coding for the niacin receptor 1, was also induced after surgical incision, and agonism of the *Niacr1* protein product can inhibit proinflammatory cytokine production.[54] Other GPCRs like *Gpr183*, a GPCR with constitutive activity,[55] and *Gpr31*, the receptor for 12(S)-hydroxyeicosatetranoic acid,[56] have not yet been thoroughly assessed for their role in pain states. Further work is needed to determine the role of these transcriptional changes in the sensitization process, and whether pharmacologic manipulation of these receptors could alter postsurgical hypersensitivity. Genes encoding phospholipase D4, phospholipase A2 group IVA, arachidonate 5-lipoxygenase, and thromboxane A synthase 1 were upregulated after surgical incision. These changes implicate a strong role for multiple inflammatory lipid mediators that may act in conjunction with prostaglandins. The full list of differentially expressed genes between

any pairwise comparison of our experimental groups can be found in Supplemental Digital Content 13 (http://links.lww.com/ALN/B587).

Interestingly, despite providing robust analgesia, preemptive resiniferatoxin failed to attenuate many genes upregulated by surgical incision (fig. 4, D and E). One exception to this pattern is the prodynorphin gene (*Pdyn*), the expression of which is not significantly increased after incision with resiniferatoxin compared to 118.4% after incision without resiniferatoxin (fig. 6A). *Pdyn* encodes the precursor of the neuropeptide dynorphin, which is currently hypothesized to have a role in facilitating chronic pain (see recent review[57]) and is known to be upregulated in the DH after inflammation, nerve injury, and surgical incision.[58–60] The expression of the opioid receptors did not change after surgical incision (table 1), consistent with previous work showing no changes in μ, δ, or κ opioid receptor expression or binding after inflammation or nerve injury.[58,61] Our results suggest that the partial attenuation of *Pdyn* in the resiniferatoxin-treated group could be a correlate of reduced nociceptive physiologic inputs as a result of resiniferatoxin analgesia.

As with surgical incision without analgesia, surgical incision with resiniferatoxin induced many transcriptomic changes that are predominantly related to microglia activation. Microglial activation is likely induced from altered trophic signaling from damaged primary afferents.[34] Markers of nerve injury are induced in the dorsal root ganglia after surgical incision, such as the transcription factor ATF3,[62] and intraplantar resiniferatoxin also induces ATF3 in the dorsal root ganglia,[38,63] consistent with the idea of resiniferatoxin-induced calcium overload leading to nerve terminal/axon damage.[14] If incisional nerve injury at the peripheral terminal triggers central changes that lead to microglial activation, then it is not surprising that resiniferatoxin fails to abrogate these changes. Indeed, intraplantar resiniferatoxin without surgical incision trended toward modest, although nonsignificant, microglial activation in the DH (Supplemental Digital Content 12, http://links.lww.com/ALN/B586), consistent with previous work showing microglial proliferation after perineural resiniferatoxin.[64] While there are components of axonal damage after surgical incision, this intervention alters transcriptional networks in a distinct way from neuropathic pain models such as the spared nerve injury model, although the few shared genes induced by both conditions are largely microglial-specific[65] (Supplemental Digital Content 11H, http://links.lww.com/ALN/B585).

The persistence of transcriptomic changes in the central nervous system despite robust postsurgical analgesia with resiniferatoxin is surprising. With the possible exception of *Pdyn*, transcriptomic induction in the postsurgical DH does not appear to be due to postsurgical hyperalgesia *per se*, and thus resiniferatoxin can effectively block one without preventing the other. This raises the hypothesis that postsurgical hyperalgesia is caused by at least two mechanisms. First, TRPV1$^+$ fibers are required for the induction and maintenance of postincisional hyperalgesia, which is blocked after peripheral injection of resiniferatoxin. Second, surgical injury to multiple fiber types including TRPV1$^-$ large A-fibers[64] leads to microglial activation and proliferation (fig. 7), and when TRPV1$^+$ nociceptor signaling is intact, microglia activation contributes to postsurgical pain.[48,66] In conclusion, local preemptive administration of resiniferatoxin has many of the characteristics of a clinically useful perioperative analgesic: the inhibition of nociceptive stimulation and hyperalgesia is broadly effective, motoric function is unaffected, and a single injection circumscribes the most painful postoperative time period. As skin is reinnervated by both thermal C-fibers and A-δ fibers, the nerve terminal inactivation with peripherally administered resiniferatoxin is reversed.[38] Our work emphasizes the importance of TRPV1$^+$ fibers in postsurgical nocifensive behavior, and highlights that analgesia is possible through TRPV1$^+$ fiber inactivation despite central changes in the postsurgical DH.

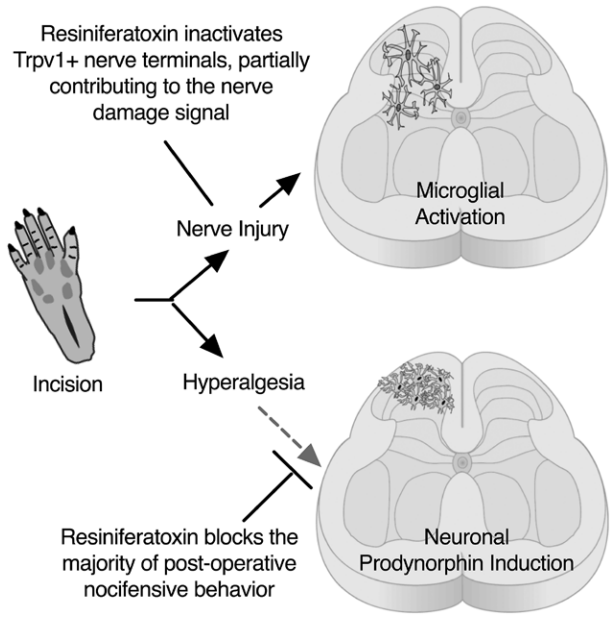

Fig. 7. Proposed model for transcriptomic changes after surgical incision. Incision leads to nerve injury as well as tissue damage and local inflammation. Nerve injury is likely responsible for microglial activation, which resiniferatoxin (RTX) fails to attenuate. In contrast, resiniferatoxin blocks the majority of postoperative nocifensive behaviors, a possible molecular correlate of which is the prodynorphin transcript level, which is induced by incision and has attenuated induction with resiniferatoxin treatment.

Acknowledgments

The authors would like to thank Amelia Loydpierson, B.A., Department of Perioperative Medicine, Clinical Center, National Institutes of Health (NIH), Bethesda, Maryland, for her contributions to the immunohistochemical staining. Stephen J. Raithel, B.A., was supported by the NIH Medical Research Scholars Program, a public–private partnership supported jointly by the

NIH and generous contributions to the Foundation for the NIH from the Doris Duke Charitable Foundation (New York, New York), the American Association for Dental Research (Alexandria, Virginia), the Colgate-Palmolive Company (New York, New York), Genentech (San Francisco, California), alumni of student research programs, and other individual supporters. The authors also acknowledge the early support of the National Center for Complementary and Integrative Health, NIH, Bethesda, Maryland.

Research Support

This work was supported by the intramural research program of the Clinical Center, National Institutes of Health (Bethesda, Maryland).

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Iadarola: Department of Perioperative Medicine, Clinical Center, Building 10, Room 2C401, 10 Center Drive, MSC 1510, National Institutes of Health, Bethesda, Maryland 20892–1510. miadarol@cc.nih.gov. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

1. Gan TJ, Habib AS, Miller TE, White W, Apfelbaum JL: Incidence, patient satisfaction, and perceptions of post-surgical pain: Tesults from a US national survey. Curr Med Res Opin 2014; 30:149–60
2. Sommer M, de Rijke JM, van Kleef M, Kessels AG, Peters ML, Geurts JW, Gramke HF, Marcus MA: The prevalence of postoperative pain in a sample of 1490 surgical inpatients. Eur J Anaesthesiol 2008; 25:267–74
3. Morrison RS, Magaziner J, McLaughlin MA, Orosz G, Silberzweig SB, Koval KJ, Siu AL: The impact of post-operative pain on outcomes following hip fracture. Pain 2003; 103:303–11
4. Pluijms WA, Steegers MA, Verhagen AF, Scheffer GJ, Wilder-Smith OH: Chronic post-thoracotomy pain: A retrospective study. Acta Anaesthesiol Scand 2006; 50:804–8
5. Poleshuck EL, Katz J, Andrus CH, Hogan LA, Jung BF, Kulick DI, Dworkin RH: Risk factors for chronic pain following breast cancer surgery: A prospective study. J Pain 2006; 7:626–34
6. Buvanendran A, Kroin JS: Multimodal analgesia for controlling acute postoperative pain. Curr Opin Anaesthesiol 2009; 22:588–93
7. Woolf CJ: Central sensitization: Implications for the diagnosis and treatment of pain. Pain 2011; 152(suppl 3):S2–15
8. Bamigboye AA, Hofmeyr GJ: Local anaesthetic wound infiltration and abdominal nerves block during caesarean section for postoperative pain relief. Cochrane Database Syst Rev 2009; CD006954, doi:10.1002/14651858.CD006954.pub2
9. Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D: Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 2000; 288:306–13
10. Mitchell K, Lebovitz EE, Keller JM, Mannes AJ, Nemenov MI, Iadarola MJ: Nociception and inflammatory hyperalgesia evaluated in rodents using infrared laser stimulation after Trpv1 gene knockout or resiniferatoxin lesion. Pain 2014; 155:733–45
11. Banik RK, Brennan TJ: Trpv1 mediates spontaneous firing and heat sensitization of cutaneous primary afferents after plantar incision. Pain 2009; 141:41–51
12. Brown JD, Saeed M, Do L, Braz J, Basbaum AI, Iadarola MJ, Wilson DM, Dillon WP: CT-guided injection of a TRPV1 agonist around dorsal root ganglia decreases pain transmission in swine. Sci Transl Med 2015; 7:305ra145
13. Brown DC, Agnello K, Iadarola MJ: Intrathecal resiniferatoxin in a dog model: Efficacy in bone cancer pain. Pain 2015; 156:1018–24
14. Neubert JK, Karai L, Jun JH, Kim HS, Olah Z, Iadarola MJ: Peripherally induced resiniferatoxin analgesia. Pain 2003; 104:219–28
15. Kárai LJ, Russell JT, Iadarola MJ, Oláh Z: Vanilloid receptor 1 regulates multiple calcium compartments and contributes to Ca2+-induced Ca2+ release in sensory neurons. J Biol Chem 2004; 279:16377–87
16. Karai L, Brown DC, Mannes AJ, Connelly ST, Brown J, Gandal M, Wellisch OM, Neubert JK, Olah Z, Iadarola MJ: Deletion of vanilloid receptor 1-expressing primary afferent neurons for pain control. J Clin Invest 2004; 113:1344–52
17. Tverskoy M, Cozacov C, Ayache M, Bradley EL Jr, Kissin I: Postoperative pain after inguinal herniorrhaphy with different types of anesthesia. Anesth Analg 1990; 70:29–35
18. Pogatzki-Zahn EM, Shimizu I, Caterina M, Raja SN: Heat hyperalgesia after incision requires TRPV1 and is distinct from pure inflammatory pain. Pain 2005; 115:296–307
19. Honore P, Wade CL, Zhong C, Harris RR, Wu C, Ghayur T, Iwakura Y, Decker MW, Faltynek C, Sullivan J, Jarvis MF: Interleukin-1αβ gene-deficient mice show reduced nociceptive sensitivity in models of inflammatory and neuropathic pain but not post-operative pain. Behav Brain Res 2006; 167:355–64
20. Kroin JS, Buvanendran A, Nagalla SK, Tuman KJ: Postoperative pain and analgesic responses are similar in male and female Sprague-Dawley rats. Can J Anaesth 2003; 50:904–8
21. Brennan TJ, Vandermeulen EP, Gebhart GF: Characterization of a rat model of incisional pain. Pain 1996; 64:493–501
22. Alexandre C, Latremoliere A, Ferreira A, Miracca G, Yamamoto M, Scammell TE, Woolf CJ: Decreased alertness due to sleep loss increases pain sensitivity in mice. Nat Med 2017; 23:768–74
23. Xu J, Brennan TJ: Guarding pain and spontaneous activity of nociceptors after skin *versus* skin plus deep tissue incision. ANESTHESIOLOGY 2010; 112:153–64
24. Goswami SC, Mishra SK, Maric D, Kaszas K, Gonnella GL, Clokie SJ, Kominsky HD, Gross JR, Keller JM, Mannes AJ, Hoon MA, Iadarola MJ: Molecular signatures of mouse TRPV1-lineage neurons revealed by RNA-Seq transcriptome analysis. J Pain 2014; 15:1338–59
25. Zhang W, Yu Y, Hertwig F, Thierry-Mieg J, Zhang W, Thierry-Mieg D, Wang J, Furlanello C, Devanarayan V, Cheng J, Deng Y, Hero B, Hong H, Jia M, Li L, Lin SM, Nikolsky Y, Oberthuer A, Qing T, Su Z, Volland R, Wang C, Wang MD, Ai J, Albanese D, Asgharzadeh S, Avigad S, Bao W, Bessarabova M, Brilliant MH, Brors B, Chierici M, Chu T-M, Zhang J, Grundy RG, He MM, Hebbring S, Kaufman HL, Lababidi S, Lancashire LJ, Li Y, Lu XX, Luo H, Ma X, Ning B, Noguera R, Peifer M, Phan JH, Roels F, Rosswog C, Shao S, Shen J, Theissen J, Tonini GP, Vandesompele J, Wu P-Y, Xiao W, Xu J, Xu W, Xuan J, Yang Y, Ye Z, Dong Z, Zhang KK, Yin Y, Zhao C, Zheng Y, Wolfinger RD, Shi T, Malkas LH, Berthold F, Wang J, Tong W, Shi L, Peng Z, Fischer M: Comparison of RNA-seq and microarray-based models for clinical endpoint prediction. Genome Biol 2015; 16:133
26. LaPaglia DM, Sapio MR, Burbelo PD, Thierry-Mieg J, Thierry-Mieg D, Raithel SJ, Ramsden CE, Iadarola MJ, Mannes AJ: RNA-seq investigations of human post-mortem trigeminal ganglia. Cephalalgia 2017; 0333102417720216, doi:10.1177/0333102417720216

27. Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O'Keeffe S, Phatnani HP, Guarnieri P, Caneda C, Ruderisch N, Deng S, Liddelow SA, Zhang C, Daneman R, Maniatis T, Barres BA, Wu JQ: An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. J Neurosci 2014; 34:11929–47
28. Reimand J, Arak T, Adler P, Kolberg L, Reisberg S, Peterson H, Vilo J: g:Profiler-a web server for functional interpretation of gene lists (2016 update). Nucleic Acids Res 2016; 44(W1):W83–9
29. Wall PD, Devor M, Inbal R, Scadding JW, Schonfeld D, Seltzer Z, Tomkiewicz MM: Autotomy following peripheral nerve lesions: Experimental anaesthesia dolorosa. Pain 1979; 7:103–11
30. Blumenkopf B, Lipman JJ: Studies in autotomy: Its pathophysiology and usefulness as a model of chronic pain. Pain 1991; 45:203–9
31. Wong K, Noubade R, Manzanillo P, Ota N, Foreman O, Hackney JA, Friedman BA, Pappu R, Scearce-Levie K, Ouyang W: Mice deficient in NRROS show abnormal microglial development and neurological disorders. Nat Immunol 2017; 18:633–41
32. Buttgereit A, Lelios I, Yu X, Vrohlings M, Krakoski NR, Gautier EL, Nishinakamura R, Becher B, Greter M: Sall1 is a transcriptional regulator defining microglia identity and function. Nat Immunol 2016; 17:1397–406
33. Bédard A, Tremblay P, Chernomoretz A, Vallières L: Identification of genes preferentially expressed by microglia and upregulated during cuprizone-induced inflammation. Glia 2007; 55:777–89
34. Guan Z, Kuhn JA, Wang X, Colquitt B, Solorzano C, Vaman S, Guan AK, Evans-Reinsch Z, Braz J, Devor M, Abboud-Werner SL, Lanier LL, Lomvardas S, Basbaum AI: Injured sensory neuron-derived CSF1 induces microglial proliferation and DAP12-dependent pain. Nat Neurosci 2016; 19:94–101
35. Kissin I, Davison N, Bradley EL Jr: Perineural resiniferatoxin prevents hyperalgesia in a rat model of postoperative pain. Anesth Analg 2005; 100:774–80
36. Neubert JK, Mannes AJ, Karai LJ, Jenkins AC, Zawatski L, Abu-Asab M, Iadarola MJ: Perineural resiniferatoxin selectively inhibits inflammatory hyperalgesia. Mol Pain 2008; 4:3
37. Kang SC, Jampachaisri K, Seymour TL, Felt SA, Pacharinsak C: Use of liposomal bupivacaine for postoperative analgesia in an incisional pain model in rats (Rattus norvegicus). J Am Assoc Lab Anim Sci 2017; 56:63–8
38. Mitchell K, Bates BD, Keller JM, Lopez M, Scholl L, Navarro J, Madian N, Haspel G, Nemenov MI, Iadarola MJ: Ablation of rat TRPV1-expressing Adelta/C-fibers with resiniferatoxin: Analysis of withdrawal behaviors, recovery of function and molecular correlates. Mol Pain 2010; 6:94
39. Wu C, Gavva NR, Brennan TJ: Effect of AMG0347, a transient receptor potential type V1 receptor antagonist, and morphine on pain behavior after plantar incision. Anesthesiology 2008; 108:1100–8
40. Uchytilova E, Spicarova D, Palecek J: TRPV1 antagonist attenuates postoperative hypersensitivity by central and peripheral mechanisms. Mol Pain 2014; 10:67
41. Honore P, Wismer CT, Mikusa J, Zhu CZ, Zhong C, Gauvin DM, Gomtsyan A, El Kouhen R, Lee CH, Marsh K, Sullivan JP, Faltynek CR, Jarvis MF: A-425619 [1-isoquinolin-5-yl-3-(4-trifluoromethyl-benzyl)-urea], a novel transient receptor potential type V1 receptor antagonist, relieves pathophysiological pain associated with inflammation and tissue injury in rats. J Pharmacol Exp Ther 2005; 314:410–21
42. Kang S, Wu C, Banik RK, Brennan TJ: Effect of capsaicin treatment on nociceptors in rat glabrous skin one day after plantar incision. Pain 2010; 148:128–40
43. Hamalainen MM, Subieta A, Arpey C, Brennan TJ: Differential effect of capsaicin treatment on pain-related behaviors after plantar incision. J Pain 2009; 10:637–45
44. Pospisilova E, Palecek J: Post-operative pain behavior in rats is reduced after single high-concentration capsaicin application. Pain 2006; 125:233–43
45. Cavanaugh DJ, Lee H, Lo L, Shields SD, Zylka MJ, Basbaum AI, Anderson D.J: Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. Proc Natl Acad Sci USA 2009; 106:9075–80
46. Zhuo M, Wu G, Wu LJ: Neuronal and microglial mechanisms of neuropathic pain. Mol Brain 2011; 4:31
47. Inoue K, Tsuda M: Microglia and neuropathic pain. Glia 2009; 57:1469–79
48. Wen YR, Suter MR, Ji RR, Yeh GC, Wu YS, Wang KC, Kohno T, Sun WZ, Wang CC: Activation of p38 mitogen-activated protein kinase in spinal microglia contributes to incision-induced mechanical allodynia. Anesthesiology 2009; 110:155–65
49. Verge GM, Milligan ED, Maier SF, Watkins LR, Naeve GS, Foster AC: Fractalkine (CX3CL1) and fractalkine receptor (CX3CR1) distribution in spinal cord and dorsal root ganglia under basal and neuropathic pain conditions. Eur J Neurosci 2004; 20:1150–60
50. Milligan ED, Zapata V, Chacur M, Schoeniger D, Biedenkapp J, O'Connor KA, Verge GM, Chapman G, Green P, Foster AC, Naeve GS, Maier SF, Watkins LR: Evidence that exogenous and endogenous fractalkine can induce spinal nociceptive facilitation in rats. Eur J Neurosci 2004; 20:2294–302
51. Griffin RS, Costigan M, Brenner GJ, Ma CH, Scholz J, Moss A, Allchorne AJ, Stahl GL, Woolf CJ: Complement induction in spinal cord microglia results in anaphylatoxin C5a-mediated pain hypersensitivity. J Neurosci 2007; 27:8699–708
52. Barragán-Iglesias P, Pineda-Farias JB, Cervantes-Durán C, Bravo-Hernández M, Rocha-González HI, Murbartián J, Granados-Soto V: Role of spinal P2Y6 and P2Y11 receptors in neuropathic pain in rats: possible involvement of glial cells. Mol Pain 2014; 10:29
53. Nicol LS, Dawes JM, La Russa F, Didangelos A, Clark AK, Gentry C, Grist J, Davies JB, Malcangio M, McMahon SB: The role of G-protein receptor 84 in experimental neuropathic pain. J Neurosci 2015; 35:8959–69
54. Parodi B, Rossi S, Morando S, Cordano C, Bragoni A, Motta C, Usai C, Wipke BT, Scannevin RH, Mancardi GL, Centonze D, Kerlero de Rosbo N, Uccelli A: Fumarates modulate microglia activation through a novel HCAR2 signaling pathway and rescue synaptic dysregulation in inflamed CNS. Acta Neuropathol 2015; 130:279–95
55. Rosenkilde MM, Benned-Jensen T, Andersen H, Holst PJ, Kledal TN, Lüttichau HR, Larsen JK, Christensen JP, Schwartz TW: Molecular pharmacological phenotyping of EBI2. An orphan seven-transmembrane receptor with constitutive activity. J Biol Chem 2006; 281:13199–208
56. Honn KV, Guo Y, Cai Y, Lee MJ, Dyson G, Zhang W, Tucker SC: 12-HETER1/GPR31, a high-affinity 12(S)-hydroxyeicosatetraenoic acid receptor, is significantly up-regulated in prostate cancer and plays a critical role in prostate cancer progression. FASEB J 2016; 30:2360–9
57. Podvin S, Yaksh T, Hook V: The emerging role of spinal dynorphin in chronic pain: A therapeutic perspective. Annu Rev Pharmacol Toxicol 2016; 56:511–33
58. Iadarola MJ, Brady LS, Draisci G, Dubner R: Enhancement of dynorphin gene expression in spinal cord following experimental inflammation: Stimulus specificity, behavioral parameters and opioid receptor binding. Pain 1988; 35:313–26
59. Kajander KC, Sahara Y, Iadarola MJ, Bennett GJ: Dynorphin increases in the dorsal spinal cord in rats with a painful peripheral neuropathy. Peptides 1990; 11:719–28
60. Zhu X, Vincler MA, Parker R, Eisenach JC: Spinal cord dynorphin expression increases, but does not drive microglial prostaglandin production or mechanical hypersensitivity after incisional surgery in rats. Pain 2006; 125:43–52
61. Obara I, Parkitna JR, Korostynski M, Makuch W, Kaminska D, Przewlocka B, Przewlocki R: Local peripheral opioid effects

and expression of opioid genes in the spinal cord and dorsal root ganglia in neuropathic and inflammatory pain. Pain 2009; 141:283–91

62. Hill CE, Harrison BJ, Rau KK, Hougland MT, Bunge MB, Mendell LM, Petruska JC: Skin incision induces expression of axonal regeneration-related genes in adult rat spinal sensory neurons. J Pain 2010; 11:1066–73
63. Lebovitz EE, Keller JM, Kominsky H, Kaszas K, Maric D, Iadarola MJ: Positive allosteric modulation of TRPV1 as a novel analgesic mechanism. Mol Pain 2012; 8:70
64. Suter MR, Berta T, Gao YJ, Decosterd I, Ji RR: Large A-fiber activity is required for microglial proliferation and p38 MAPK activation in the spinal cord: Different effects of resiniferatoxin and bupivacaine on spinal microglial changes after spared nerve injury. Mol Pain 2009; 5:53
65. Costigan M, Moss A, Latremoliere A, Johnston C, Verma-Gandhu M, Herbert TA, Barrett L, Brenner GJ, Vardeh D, Woolf CJ, Fitzgerald M: T-cell infiltration and signaling in the adult dorsal spinal cord is a major contributor to neuropathic pain-like hypersensitivity. J Neurosci 2009; 29:14415–22
66. Peters CM, Eisenach JC: Contribution of the chemokine (C-C motif) ligand 2 (CCL2) to mechanical hypersensitivity after surgical incision in rats. ANESTHESIOLOGY 2010; 112:1250–8

ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

Slocum's Cocaine "Surprise" for Johnson Pillmore, M.D.

From the Wood Library-Museum's Ben Z. Swanson Collection, this illegibly postmarked cover (*above*) was sent to Johnson Pillmore, M.D., of Delta, New York. Fortunately, the "Columbus in Sight of Land" 1-cent stamp from the Columbian Issue assists us in dating this item. From Holly, Michigan, the Slocum Manufacturing Company was taking advantage of the 1893 World's Columbian Exposition to advertise "Dental Surprise," their new (?) "local anesthetic for painless extraction of teeth." Suspicious of the novelty of Slocum's wares, the journal editor of the *Dental Cosmos* forwarded a sample of "Dental Surprise" in 1893 to S. P. Sadtler, Ph.D., a professor at the Philadelphia College of Pharmacy. Sadtler's chemical analysis unmasked "Dental Surprise" as merely carbolic acid fortified with 1.46% "Anhydrous Cocain Hydrochlorate." (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

George S. Bause, M.D., M.P.H., Honorary Curator and Laureate of the History of Anesthesia, Wood Library-Museum of Anesthesiology, Schaumburg, Illinois, and Clinical Associate Professor, Case Western Reserve University, Cleveland, Ohio. UJYC@aol.com.