Individual Differences in Acute Pain-induced Endogenous Analgesia Predict Time to Resolution of Postoperative Pain in the Rat

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ABSTRACT

Background: Chronic postsurgical pain, a significant public health problem, occurs in 10 to 50% of patients undergoing major surgery. Acute pain induces endogenous analgesia termed conditioned pain modulation (CPM), and the strength of CPM preoperatively predicts the likelihood of chronic postsurgical pain. The relation between CPM and recovery from surgery has not been examined in preclinical models.

Methods: CPM was assessed in individual rats and correlated with each animal’s time course of recovery of hypersensitivity after partial spinal nerve ligation. The role of descending noradrenergic pathways in the spinal cord to mechanisms of CPM and recovery was tested using idazoxan to block noradrenergic receptors or antidopamine β-hydroxylase–conjugated saporin to ablate these pathways. Behavioral hypersensitivity, static weight bearing, and spinal glial activation were measured after partial spinal nerve ligation.

Results: The strength of CPM varied over two-fold between individuals and was directly correlated with the slope of recovery from hypersensitivity after surgery ($P < 0.0001; r = 0.660$). CPM induced the release of norepinephrine in the spinal cord and was partially blocked by intrathecal idazoxan or dopamine β-hydroxylase-saporin. Dopamine β-hydroxylase-saporin also slowed recovery and enhanced spinal glial activation after partial spinal nerve ligation surgery. Ongoing activation of these pathways was critical to sustained recovery because intrathecal dopamine β-hydroxylase-saporin given 7 weeks after recovery reinstated hypersensitivity, while having no effect in animals without previous surgery.

Conclusion: Collectively, these studies provide a clear back-translation from clinical observations of CPM and chronic postsurgical pain and suggest that the ability to engage ongoing descending endogenous noradrenergic signaling may be critical in determining time course of recovery from hypersensitivity after surgery. (Anesthesiology 2015; 122:895-907)
Central nervous system responses to nerve injury that facilitate pain, including spinal neural and glial activation and descending facilitation, have been the focus of neuroscience research for the past 15 yr, yet their relevance to the clinical condition is unknown. Descending inhibition, in contrast, has been less studied, yet the relation between CPM and CPSP observed clinically suggests a clear relevance. Acute pain activates pontospinal noradrenergic pathways resulting in antinociception in acute, incisional, and inflammatory pain models although the role of this pathway in CPM has been less examined. Disruption of noradrenergic neurotransmission may increase or decrease hypersensitivity from nerve injury depending on which projections are ablated. We hypothesized that CPM depends at least in part on descending noradrenergic inhibition and that recovery from the acute and sustained pain after surgery might mechanistically depend on this pathway.

Finally, recovery of normal behavior after several types of stress reflects a new homeostasis from a balance of counteracting mechanisms. Several studies suggest that the transition from acute to chronic pain after injury involves a disruption in the balance of homeostatic mechanisms with a decrease in inhibitory and an increase in facilitatory (excitatory) influences at multiple levels of the neuroaxis. Alternatively, resolution of pain from tissue injury might involve a more complex up-regulation of both inhibitory and facilitatory mechanisms. For example, inflammation and injury often induce pain facilitation which is latent in the absence of a subsequent injury, as exemplified by inflammation-induced hyperalgesic priming in primary sensory afferents or latent sensitization of spinal cord circuits after inflammation and injury-induced spinal release of endogenous opioids. Inhibitory systems, which counterbalance these excitatory responses after injury, have received less attention. We hypothesize that recovery from hypersensitivity after peripheral nerve injury might involve a new homeostasis requiring ongoing activation of descending noradrenergic tone.

Materials and Methods

Animal Procedures
A total of 113 male Sprague–Dawley rats (Harlan Industries, Indianapolis, IN), weighing 250 to 300 g, were used for the experiments. All studies conformed to the Wake Forest University Guidelines on the ethical use of animals, and studies were performed under Animal Care and Use Committee (Winston-Salem, North Carolina) approval. Animals were housed under a 12-h light–dark cycle, with food and water ad libitum.

Drugs
For studies involving CPM, capsaicin (8-methyl-N-vanillyl-6-nonenamide; Sigma Chemical Co., St. Louis, MO) was prepared fresh in a vehicle solution of 10% ethanol, 10% Tween-80, and 80% sterile saline to a concentration of 3 mg/mL. Idazoxan (Tocris Bioscience, Bristol, United Kingdom) was prepared in sterile saline and passed through a 0.2 μm filter. All intrathecal drug injections were made via percutaneous lumbar puncture. Successful puncture of the dura was confirmed by the presence of a tail flick. Antidopamine β-hydroxylase conjugated to saporin (DBH-saporin) and control immunoglobulin G (IgG)-saporin were obtained from Advanced Targeting Systems, San Diego, CA, and injected in a dose of 5 μg, intrathecally.

Assessment of CPM
Conditioned pain modulation was assessed using a previously described procedure developed in rats. CPM was elicited by subdermal injection of capsaicin (150 μg in a volume of 50 μl) administered on a forepaw during brief isoflurane (3.5%) general anesthesia. This procedure produces a transient, intense stimulation of transient receptor potential vanilloid-1-expressing sensory afferents resulting in local irritation and inflammation of the injected paw as well as a more prolonged widespread analgesia of regions of the body not impacted by the injection. The local, algesic effect of capsaicin lasts several minutes, whereas the analgesia lasts approximately 1 h. The degree of analgesia in the hind paw was assessed using the Randall–Selitto paw pressure test. At each time point, paw pressure thresholds were assessed twice on the plantar aspect of both hind paws, and the results were averaged. For pharmacological studies, spinal drugs were administered 5 min before injection of capsaicin into the forepaw. For ablative studies, rats were randomly assigned to treatment groups and DBH-saporin or IgG-saporin was delivered 14 days before CPM testing. A total of 20 rats were assessed for CPM before peripheral nerve injury for individual trajectory of recovery studies; however, one rat died after spinal nerve ligation and was excluded from correlative analysis.

Partial L5 Spinal Nerve Ligation
We chose partial spinal nerve ligation (pSNL) to study recovery because this surgical injury has previously been shown to produce a transient mechanical hypersensitivity that resolves by 5 to 10 weeks postsurgery. It also has a nerve injury component which may better reflect the incidental nerve injury that accompanies some types of surgical procedures in humans. A 3-cm incision was made along the right dorsal surface near the spine using aseptic conditions, penetrating underlying muscles. The sixth lumbar transverse process was removed, and the caudal 1/3 of the L5 spinal nerve was ligated using 8-0 nylon sutures (Ethicon catalog # BV130-4, Somerville, NJ).

Assessment of Mechanical Sensitivity
Paw withdrawal thresholds to mechanical stimuli were determined using von Frey filament application. In brief, rats were placed in individual clear acrylic chambers with a plastic mesh floor and allowed to acclimate to the test environment.
at least 30 min before testing. Filaments were applied to the bending point for 6 s, and a brisk paw withdrawal was considered a positive response. Withdrawal threshold was determined using an up–down statistical method.22

Incapacitance Testing
Static weight bearing was measured using an incapacitance apparatus (Linton Instrumentation, Norfolk, United Kingdom). A clear acrylic box with an inclined plane was placed over the two footpads allowing independent measurement of the weight that the rat applied on each hindlimb. Rats were acclimated to the apparatus a minimum of three times before surgery. Static weight bearing was assessed before pSNL and for several days after surgery (postsurgical days 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20). A minimum of three readings were obtained for each paw, and the values were averaged for each paw. In the absence of hindlimb injury, rats typically apply an equal weight on both hindlimbs.

Impact of Spinal Depletion of Noradrenergic Fibers on CPM and Recovery from pSNL
We used anti-DβH-saporin to ablate spinal noradrenergic fibers. Anti-DβH-saporin binds to the membrane-bound form of DβH when it is transiently exposed to the extracellular space during norepinephrine release.23,24 Anti-DβH-saporin–containing vesicles are internalized and retrogradely transported to the cell body where the ribosome-inactivating protein, saporin, disrupts protein synthesis leading to cell death.25 Previous studies have shown that complete ablation of spinally projecting locus coeruleus (LC) neurons occurs 7 to 14 days after intrathecal injection of anti-DβH-saporin.10,26 The dose of anti-DβH-saporin used in the current study resulted in more than 95% depletion of spinal noradrenergic fibers.10,26,27

For CPM studies, rats were randomized to receive spinal injection of anti-DβH-saporin (5 µg, intrathecally, n = 8) or control IgG-saporin (5 µg, intrathecally, n = 8) 14 days before testing. After behavioral analysis, spinal cord tissue was collected and processed for immunohistochemistry to verify depletion of noradrenergic fibers. For postoperative recovery studies, rats were randomized to receive spinal injection of anti-DβH-saporin (5 µg, intrathecally, n = 7/8, one rat died due to postsurgical complications) or control IgG-saporin (5 µg, intrathecally, n = 8) 14 days before pSNL. Mechanical withdrawal thresholds were obtained on rats before pSNL and for several days after surgery (postsurgical days 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 24, 28, 35, 42, 49, 56, 63, and 70) in the ipsilateral and contralateral paws. Rats were examined for shifts in weight bearing on the same days before assessing mechanical withdrawal thresholds. The person conducting behavior was blinded to treatment group. After behavioral analysis, spinal cord tissue was collected and processed for immunohistochemical analysis to examine glial activation and verify depletion of noradrenergic fibers (see Immunohistochemistry). In a third experiment to determine whether disruption of spinal noradrenergic fibers reinstates mechanical hypersensitivity in recovered rats, mechanical withdrawal thresholds were assessed before pSNL and for several days after surgery. Beginning 91 days after pSNL, when mechanical hypersensitivity had resolved, rats were randomized to receive injections of DβH-saporin (5 µg, intrathecally, n = 6) or control IgG-saporin (5 µg, intrathecally, n = 6) and were assessed behaviorally 7 and 14 days after injection. After final behavioral analysis (106 days after pSNL), spinal cord tissue was collected and analyzed for norepinephrine content to verify depletion of noradrenergic fibers. Values were also compared with norepinephrine content from spinal cord of normal rats (n = 3) and pSNL rats 21 days after surgery (n = 6).

Relation between CPM and Postoperative Recovery of Mechanical Hypersensitivity from pSNL
In separate experiments, we used 19 of 20 rats (one rat died due to postsurgical complications) to examine the relation between preoperative CPM and postoperative recovery from hypersensitivity. Mechanical withdrawal thresholds were determined 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 24, 28, 35, 42, 49, 56, 63, and 70 days after pSNL surgery in the ipsilateral and contralateral paws. Behavioral assessment of preoperative CPM and postoperative trajectory of recovery from mechanical hypersensitivity were conducted by independent investigators so that the assessment of postoperative recovery could be completed in a blinded manner. Growth curve modeling was applied to individual rat’s mechanical withdrawal thresholds over time to obtain a trajectory of recovery for comparison to preoperative CPM responses (see Statistical Analysis).

Immunohistochemistry
Spinal cords were collected 70 days after pSNL in rats previously administered DβH-saporin (5 µg, intrathecally, n = 7) or control IgG-saporin (5 µg, intrathecally, n = 8) from the behavioral studies mentioned above. In brief, rats were anesthetized with sodium pentobarbital (intraperitoneal injection; 100 mg/kg), the thorax was opened, and 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by fixative (4% paraformaldehyde in 0.1 M PBS, pH 7.4) was perfused through the left ventricle with a peristaltic pump (20 ml/min). The spinal cord was removed and immersed in fixative for 12 h at 4°C followed by immersion in 30% sucrose at 4°C for cryoprotection until ready to be sectioned. Spinal cord cross sections (40 µm) were cut on a cryostat. For labeling noradrenergic fibers, an antibody against DβH (mouse anti-rat DβH; Millipore, Billerica, MA) was used. For assessing spinal glial activation, an antibody against glial fibrillary acidic protein (GFAP; mouse anti-rat GFAP; Sigma-Aldrich) was used to label astrocytes and an antibody against ionized calcium-binding adapter molecule 1 (IBA1, rabbit anti-rat IBA1; Wako, Richmond, VA) to label microglia. Spinal cord sections were processed free floating and incubated over night at 4°C with primary antibody. Primary antibodies were diluted in a solution consisting of PBS
containing 1% normal donkey serum and 0.1% Triton X-100 (Sigma-Aldrich). Sections were washed in PBS and incubated in appropriate secondary antisera including CY3-conjugated donkey anti-rabbit IgG (1:600; Jackson Immunoresearch, West Grove, PA) and CY2-conjugated donkey anti-mouse IgG (1:200; Jackson Immunoresearch) for 2 h at room temperature. Finally, the sections were washed thoroughly in PBS, mounted on plus-slides, air-dried, dehydrated in ethanol, cleared in xylene, and cover slipped with DPX mounting media (Sigma-Aldrich) at room temperature.

**Spinal Microdialysis and Norepinephrine Content**

On the day of experiment, anesthesia was induced with 2% isoflurane and then maintained with 1.25 to 1.5% isoflurane with spontaneous ventilation during the study. The L3 to L6 level of spinal cord was exposed by the T13-L1 laminectomy. A microdialysis probe (outer diameter = 0.22 mm, inner diameter = 0.20 mm, length = 1 mm, CX-I-8-01; EICOM Co., San Diego, CA) was inserted from just lateral to the right dorsal root 1 h before the study and perfused with Ringer’s solution (1.0 μl/min). Fractions were collected every 30 min for 2.5 h starting 1 h before capsaicin injection, and the samples were kept at −80°C until assayed for norepinephrine using high-performance liquid chromatography with electrochemical detection as previously described.28

For spinal norepinephrine content, rats were killed by deep isoflurane anesthesia followed by decapitation. The spinal cord was quickly removed and the lumbar enlargement (L4 to L6) was rapidly hemisected into left and right sides on ice and then frozen in dry ice cooled 2-methylbutane. After treatment with 0.1 M perchloric acid, the spinal tissues were homogenized on ice and centrifuged. Supernatants were collected, and norepinephrine content was measured by high-pressure liquid chromatography with electrochemical detection as previously described.28

**Image Analysis and Quantification**

Tissue sections were examined with fluorescent microscopy and images of ipsilateral and contralateral L4 to L5 dorsal spinal cord were captured with a charged coupled device digital camera attached to the microscope using a 10× objective at a resolution of 1,600 × 1,200 pixels. For semiquantitative analysis of immunofluorescence levels, a square with a fixed area (250 × 250 μm²) covering the region of laminae I to II was positioned in the lateral, central, and medial aspects of the spinal cord dorsal horn. The number of pixels occupied by immunoreactive cells within a defined threshold was measured using image analysis software (Image J; NIH Image, National Institutes of Health, Bethesda, Maryland). Results from these three areas of the spinal cord were summed for each spinal cord section analyzed. Immunofluorescent measurements of IBA1-immunoreactivity (IR) and GFAP-IR were obtained from a minimum of five L4/L5 spinal cord sections per rat and these were averaged. Before imaging and quantification of spinal cord tissue, sections corresponding to individual animals were coded so that the individual performing quantification was blinded to group.

**Statistical Analysis**

Spinal immunohistochemistry and norepinephrine content data were not normally distributed and are presented as median (25th, 75th percentile). Statistical analysis of these data was performed using Mann–Whitney rank sum test or Kruskal–Wallis ANOVA on ranks with comparisons between groups conducted using Student–Newman–Keuls method, as appropriate. All other data were normally distributed and are presented as mean ± SD. Weight bearing data are expressed as percentage of weight on the paw ipsilateral to surgery. Behavioral time course data for (CPM over minutes, weight bearing after pSNL over days, and withdrawal threshold after pSNL over weeks) were analyzed using a two-way repeated-measures (RM) ANOVA, assessing main effects of treatment and time followed by Bonferroni contrasts using SigmaPlot (Version 11.0; Systat Inc., San Jose, CA). Where appropriate, corrected P values were indicated for multiple comparisons.

In addition to the traditional two-way RM ANOVA analysis, we also applied a recently described and more powerful growth curve modeling approach to define the trajectory of recovery from hypersensitivity after pSNL.29 In brief, using the PROC MIXED procedure in SAS (Version 9.2, Cary, NC), withdrawal threshold over time data were fit to a linear or a quadratic model, the choice being made using Bayesian Information Criteria. In the current group populations, the data were best fit to a linear model over time, described by an intercept (modeled withdrawal threshold immediately after pSNL surgery) and slope (linear rate of change in withdrawal threshold after pSNL surgery). The intercept and slope estimates were allowed to vary across individual rats (random effects) and as a function of treatment group or condition (fixed effects). The primary inferences of the study involve examining whether experimental condition impacts some aspect of the change process. To estimate these effects, group and intervention (anti-DβH-saporin) were entered as level-2 predictors of intercept and slope parameters (i.e., as group × parameter interaction) as previously described.29

In addition to providing a secondary analysis to compare anti-DβH-saporin to IgG-saporin groups, growth curve analysis was also used to examine the relation between CPM and time course of recovery. For the latter, we applied Pearson Product Moment correlation to the strength of preoperative CPM in individual animals to that animal’s modeled slope of recovery. All the hypothesis testing is two tailed with P value less than 0.05 considered significant.

**Results**

**CPM in Rodents Is Partially Mediated by Descending Spinal Noradrenergic Pathways**

Intradermal injection of capsaicin into the forepaw resulted in a large increase in withdrawal threshold to mechanical pressure in the hind paw in vehicle-treated animals, with...
a peak effect 30 min after capsaicin injection (fig. 1A). Two-way RM ANOVA showed a significant group × time interaction \((F_{6, 40} = 18.76; P < 0.001)\). Spinal blockade of \(\alpha_2\)-adrenergic receptors with idazoxan reduced CPM magnitude compared with saline control but did not eliminate it (fig. 1A; \(P = 0.002\)). Withdrawal threshold did not change over time in animals receiving forepaw vehicle injection instead of capsaicin (fig. 1A). In a separate group of rats, spinal ablation of noradrenergic neurons with DβH-saporin (5 \(\mu\g) delivered 14 days before testing also partially reduced CPM magnitude compared with IgG-saporin–treated controls (fig. 1B; \(P < 0.05\)). Baseline hind paw pressure thresholds before forepaw capsaicin injection were not significantly different between DβH-saporin–treated and IgG-saporin–treated rats (130.3 ± 4 g vs. 133.1 ± 3 g, \(P = 0.54\), Student t test) indicating that ablation of noradrenergic pathways does not induce a hypernociceptive state.

We also examined the release of norepinephrine in the lumbar spinal cord in response to forepaw capsaicin injection using spinal microdialysis. Norepinephrine levels were significantly increased in the lumbar spinal cord for 60 min after injection of capsaicin but not vehicle solution (fig. 1C; \(P < 0.05\)). Two-way RM ANOVA did not show significant group × time interaction \((F_{3, 27} = 2.943; P = 0.051)\); however, we did observe a main effect of group \((F_{1, 9} = 7.109; P < 0.05)\) and time \((F_{3, 9} = 3.202; P < 0.05)\).

**Disrupting Spinal Noradrenergic Fibers before Surgery Prolongs Mechanical Hypersensitivity but Not Shifts in Weight Bearing**

To determine the contribution of descending spinal noradrenergic systems to resolution of postoperative hypersensitivity, we administered DβH-saporin (5 \(\mu\g) 14 days before surgery. We conducted partial L5 spinal nerve ligation and measured paw withdrawal thresholds in the ipsilateral and contralateral paw longitudinally until 70 days postsurgery. Two-way RM ANOVA showed a significant group × time interaction \((F_{18, 233} = 5.266; P < 0.001)\) and contralateral paw withdrawal thresholds \((F_{18, 233} = 3.377; P < 0.001)\). Mean withdrawal thresholds did not differ between groups before pSNL surgery and 14 days after administration of DβH-saporin or IgG-saporin (fig. 2A; \(P = 0.948\)), indicating that depletion of spinal noradrenergic fibers did not alter baseline mechanical withdrawal thresholds similar to previous reports.\(^{10,27}\) Rats that received control IgG-saporin before surgery developed mechanical hypersensitivity in the ipsilateral paw until 35 days postsurgery (fig. 2A; \(P < 0.05\)). Contralateral paw withdrawal thresholds were transiently reduced in IgG-saporin–treated rats until day 16 postsurgery (fig. 2B; \(P < 0.05\)). In contrast, DβH-saporin–treated rats had reduced withdrawal thresholds until at least 70 days postsurgery in the ipsilateral paw as well as an earlier onset and longer duration of contralateral hypersensitivity (fig. 2B).

![Fig. 1.] Endogenous analgesia was measured in rats by assessing conditioned pain modulation. (A) Hind paw withdrawal paw pressure thresholds (test stimuli) were assessed in rats after injection of capsaicin (150 \(\mu\g/50 \mu\l) or vehicle solution into the forepaw (conditioning stimuli). Rats received spinal injections of the \(\alpha_2\)-adrenergic receptor antagonist idazoxan (30 \(\mu\g, intrathecal \[i.t.\]) or saline solution via lumbar puncture 10 min before forepaw capsaicin to examine the contribution of noradrenergic mechanisms in this model of conditioned pain modulation. Two-way ANOVA with Bonferroni multiple comparisons. Values represent mean ± SD with \(n = 6\) per group. \# \(P < 0.025\) versus precapsaicin baseline. * \(P < 0.0125\) versus capsaicin + saline group. (B) Spinal ablation of noradrenergic fibers with dopamine \(\beta\)-hydroxylase (DβH)-saporin (5 \(\mu\g, i.t.)\) 14 days before testing also partially reduced conditioned pain modulation. Student t test, * \(P < 0.05\). Values represent mean ± SD with \(n = 8\) for the immunoglobulin G (IgG)-saporin group and \(n = 8\) for the DβH-saporin–treated group. (C) Time course of spinal norepinephrine (NE) release in the lumbar region of the spinal cord after injection of capsaicin into the forepaw. Two-way ANOVA with Bonferroni multiple comparisons. Values represent mean ± SD with \(n = 6\) for the capsaicin group and \(n = 4\) for the vehicle-treated group. cap = capsaicin.
β Values represent mean ± SD with n = 7 for DβHwithin treatment group comparison to presurgery baseline value. P point comparison to pSNL + IgG-saporin values or # with Bonferroni multiple comparisons. * T o test the hypothesis that endogenous spinal norepinephrine tonically maintains resolution of mechanical hypersensitivity, group × time interaction for the percentage of weight bearing on the ipsilateral paw (fig. 2C; F10,130 = 6.5; P < 0.001). Both groups of pSNL rats previously treated with DβH-saporin or IgG-saporin demonstrated a significant reduction in weight bearing on the ipsilateral paw until 12 days after surgery. pSNL DβH-saporin treated rats had significantly greater shifts in weight bearing during earlier time points (days 1, 2, and 8) postoperatively compared with pSNL IgG-saporin–treated rats (P < 0.001).

Growth curve analysis was successfully applied to mechanical withdrawal thresholds in the ipsilateral paw of DβH-saporin and IgG-saporin pSNL rats to obtain individual (fig. 3A) and group trajectories (fig. 3B) of recovery. There was a statistically significant variance in the random effects for the intercept (P < 0.0001) and the slope (P < 0.0001), indicating that individuals varied substantially in their change processes and that further modeling of these parameters was warranted (table 1). The group trajectory of anti-DβH-saporin–treated rats was significantly reduced compared with IgG-saporin–treated rats. The group modeled trajectories had nonoverlapping 95% CIs beginning 8 days postoperatively, and the modeled trajectory of DβH-saporin–treated rats had a significantly lower predicted slope (table 1), indicating a slower or delayed recovery period. The predicted intercepts were not significantly different between groups (table 1).

**Disrupting Spinal Noradrenergic Fibers before Surgery Enhances Spinal Glial Activation after pSNL**

Spinal cord tissue was collected 70 days after pSNL in rats treated with DβH-saporin (n = 7) or IgG-saporin (n = 8) for immunohistochemical analysis to verify depletion of noradrenergic fibers and for examination of spinal glial activation (fig. 4). All rats administered spinal DβH-saporin had complete loss of noradrenergic fibers at the lumbar level of the spinal cord. DβH-IR was uniformly distributed throughout the dorsal spinal cord of IgG-saporin–treated rats 70 days after pSNL (fig. 4, C, D, and G; P < 0.05). Spinal cords from DβH-saporin–treated rats also had increased GFAP-IR in the ipsilateral (P < 0.05) and contralateral (P < 0.05) dorsal horn compared with pSNL IgG-saporin–treated rats (fig. 4, A and B). We observed a significant increase in density of IBA1-IR in the ipsilateral spinal cord of DβH-saporin–treated rats 70 days after pSNL compared with IgG-saporin–treated pSNL rats (fig. 4, C, D, and G; P < 0.05). Spinal cords from DβH-saporin–treated rats also had increased GFAP-IR in the ipsilateral (P < 0.05) and contralateral (P < 0.05) dorsal horn compared with pSNL IgG-saporin–treated rats (fig. 4, E, F, and H). Increases in IBA1-IR and GFAP-IR appeared to be more prominent in superficial laminae of pSNL DβH-saporin–treated rats. Similar to other studies,10,27 we did not observe differences in GFAP-R or IBA1-IR in norepinephrine-depleted rats that did not undergo surgery (data not shown).

**Disrupting Spinal Noradrenergic Fibers after Resolution of Mechanical Hypersensitivity after pSNL Reinstates Postoperative Mechanical Hypersensitivity**

To test the hypothesis that endogenous spinal norepinephrine

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**Fig. 2.** Time course of mechanical hypersensitivity and static weight bearing after partial spinal nerve ligation (pSNL). Rats received intrathecal treatment with dopamine β-hydroxylase (DβH)-saporin or control immunoglobulin G (IgG)-saporin 14 days before surgery and were assessed for mechanical hypersensitivity with von Frey filaments and assayed for shifts in weight bearing on the ipsilateral hind paw. Mechanical withdrawal thresholds were reduced in the ipsilateral (A) and contralateral (B) paw of DβH-saporin–treated rats. Two-way repeated-measures ANOVA with Bonferroni multiple comparisons. *P < 0.003 for within time point comparison to pSNL + IgG-saporin values or #P < 0.003 for within treatment group comparisons to presurgery baseline value. Brackets indicate the data points for which withdrawal thresholds were significantly different from presurgery values. The percentage body weight shifted to the ipsilateral paw was reduced in pSNL rats from both groups for approximately 2 weeks (C). A significantly greater shift in weight bearing was present in DβH-saporin compared with IgG-saporin–treated rats particularly at early time points. Two-way repeated-measures ANOVA with Bonferroni multiple comparisons. *P < 0.003 for within time point comparison to pSNL + IgG-saporin values or #P < 0.003 for within treatment group comparison to presurgery baseline value. Values represent mean ± SD with n = 7 for DβH-saporin and n = 8 for IgG-saporin group. i.t. = intrathecal.

In the same set of rats, we also assessed static weight bearing on the hind paws as a nonevoked measure of postoperative pain. Two-way RM ANOVA showed a significant...
we depleted noradrenergic fibers after pSNL-induced hypersensitivity resolved. We delivered DβH-saporin or IgG-saporin 91 days after pSNL (approximately 7 weeks after resolution of mechanical hypersensitivity) and assessed mechanical hypersensitivity 1 and 2 weeks later. Two-way RM ANOVA showed a significant group × time interaction for the ipsilateral paw withdrawal thresholds ($F_{22, 220} = 5.162; P < 0.001$) and for the contralateral paw withdrawal thresholds ($F_{22, 220} = 3.377; P < 0.001$). Spinal injection of DβH-saporin reinstated ipsilateral (fig. 5A) and contralateral (fig. 5B) mechanical hypersensitivity in pSNL rats 7 and 14 days after treatment (fig. 5, A and B; $P < 0.001$).

We verified depletion of spinal norepinephrine by assessing norepinephrine content in the ipsilateral and contralateral dorsal spinal cord of pSNL rats 2 weeks after treatment (106 days after pSNL). Norepinephrine content in the spinal cord of DβH-saporin–treated pSNL rats was significantly lower compared with IgG-saporin–treated pSNL rats (fig. 5C). We compared norepinephrine content at this late stage of recovery to separate group of rats without surgery and at an earlier stage of recovery. Norepinephrine content was significantly increased 3 weeks until at least 15 weeks post-pSNL compared with normal rats (fig. 5C).

**Correlation between CPM and Recovery from Hypersensitivity after pSNL**

We examined the relation between the magnitude of CPM and time course of postoperative resolution of hypersensitivity in 19 rats. Rats were tested preoperatively for CPM to assess the degree of endogenous analgesia. At 30-min postforepaw capsaicin injection, there was considerable interanimal variability in strength of CPM response, with a difference from baseline ranging from 62.5 to 140 g (fig. 6A).

We conducted pSNL surgery on these same rats and measured paw withdrawal thresholds in the ipsilateral paw longitudinally until 70 days after surgery. Actual longitudinal behavioral data of individual rats (fig. 6B) were modeled after surgery using growth curve analysis to describe trajectories of recovery from hypersensitivity (fig. 6C). The mean intercept for the 19 rats was 1.46 g, with a standard error of 0.31 g. The mean slope was 0.2497 g/day, with a standard error of 0.0202 g/day. There was a significant correlation between the individual animal’s strength of preoperative CPM and its slope of recovery ($r = 0.660; P = 0.006$) as rats with lower endogenous analgesia had slower resolution of mechanical hypersensitivity. We did not observe a significant correlation between the intercept (modeled withdrawal

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**Fig. 3.** Modeled trajectories of recovery from mechanical hypersensitivity after partial spinal nerve ligation (pSNL) in rats treated with dopamine β-hydroxylase (DβH)-saporin or control immunoglobulin G (IgG)-saporin 14 days before surgery. (A) Longitudinal behavioral measurements of mechanical paw withdrawal thresholds from the ipsilateral paw of individual rats modeled using growth curve analysis were best fit to a linear function described by an intercept (modeled withdrawal threshold immediately after pSNL surgery) and slope (linear rate of change in withdrawal threshold after pSNL surgery). (B) Group trajectories depict the mean fit for all the animals within each treatment group with 95% CIs indicated by shading.

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**Table 1.** Growth Curve Modeling of Ipsilateral Mechanical Hypersensitivity in Rats with pSNL

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<th>Parameter</th>
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<th>$P$ Value</th>
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Comparison of growth curve parameters for pSNL rats treated with DβH-saporin or IgG-saporin 14 days before pSNL surgery. Growth curve analysis of paw withdrawal thresholds (day 1−70) best fit a linear model giving rise to an intercept (hypersensitivity at time 0) and slope (linear rate of change in pain measure).

DβH = dopamine β-hydroxylase; IgG = immunoglobulin G; pSNL = partial spinal nerve ligation; REF = reference value.
Fig. 4. Representative images of noradrenergic innervation and glial activation in ipsilateral dorsal spinal cord 70 days after partial spinal nerve ligation (pSNL) in rats treated with spinal dopamine β-hydroxylase (DβH)-saporin (A, C, E) or immunoglobulin G (IgG)-saporin (B, D, E) 14 days before surgery. (A and B) Long-term depletion of spinal noradrenergic fibers is evident in the dorsal spinal cord of DβH-saporin–treated rats based on loss of DβH-immunoreactivity (IR). Ionized calcium-binding adapter molecule 1 (IBA1)-IR (C, D) and glial fibrillary acidic protein (GFAP)-IR (E, F) were increased in the superficial dorsal horn of nerve-injured rats with depletion of noradrenergic fibers. Lowest panels depict the quantification of spinal cord IBA1 (G) and GFAP-IR (H) levels. Values represent median ± 25th and 75th percentile with n = 7 for DβH-saporin and n = 8 for IgG-saporin groups. Data were analyzed with Mann–Whitney rank sum test. *P < 0.05 within side comparison to pSNL + IgG-saporin value. Scale bar in F = 200 μm. contra = contralateral; ipsi = ipsilateral.
Peters et al. threshold immediately after pSNL surgery) and slope of recovery ($r = -0.18; P = 0.07$), suggesting that greater initial hypersensitivity was not predictive of slower recovery.

Preoperative CPM testing including injection of forepaw capsaicin did not impact development or resolution of hypersensitivity in the hind paws of pSNL rats as the slopes of recovery were not statistically different compared with separate cohorts of control pSNL rats that did not undergo preoperative CPM testing. Mean slope $0.25 \pm 0.02$ g/day for rats that had preoperative CPM testing versus $0.23 \pm 0.03$ g/day for rats without preoperative CPM testing ($P = 0.52$).

**Discussion**

Better understanding of mechanisms of CPSP would guide preventive strategies, and the current study provides a new approach for such translation, including novel tools to test hypotheses to distinguish causation from association and mechanisms by which individual factors affect recovery. Our data show that rats, like humans, demonstrate an individual association between the strength of preoperative CPM and speed of recovery from pain or hypersensitivity after surgery. The results support a causal link for this association, reflected in engagement of descending noradrenergic signaling, and suggest that this increased signaling is important to time course of recovery and maintenance of the recovered state.

**Mechanisms of CPM in Rodents**

Conditioned pain modulation has been studied in rats using a variety of conditioning stimuli (intradermal capsaicin or formalin or electrical stimulation) and test stimuli (heat or mechanical pressure). We used a form of CPM developed in rats called noxious stimulation–induced analgesia. Previous studies of noxious stimulation–induced analgesia have focused primarily on supraspinal opioid and dopaminergic mechanisms. In the current study, we extend the proposed role of spinal noradrenergic activity in CPM to noxious stimulation–induced analgesia by showing that forepaw capsaicin induced a two-fold increase in norepinephrine release in the lumbar spinal cord, consistent with the known effect of noxious stimulation to induce spinal norepinephrine release and by showing that noxious stimulation–induced analgesia is partially blocked by intrathecal injection of the $\alpha_2$-adrenergic antagonist idazoxan and by ablation of descending noradrenergic innervation of the cord. The partial effect of disrupting noradrenergic signaling suggests that other descending controls are likely involved, including opioidergic, serotonergic, or oxytocinergic systems. Because idazoxan antagonizes imidazoline receptors, which can inhibit hypersensitivity in rat models of inflammatory, neuropathic pain, and incisional pain, we cannot exclude a contribution of spinal imidazoline receptors in this model of CPM.
Pharmacological blockade, genetic disruption, and toxin-mediated ablation of descending noradrenergic projections have all been applied to understand the contribution of spinal noradrenergic systems in the degree of hypersensitivity induced by nerve injury. In our study, using a surgical model with resolution of hypersensitivity over 5 to 10 weeks, preoperative depletion of spinal noradrenergic fibers delayed recovery, with separation from control animals within 1 week of the surgery. Others have shown that ablation of spinal noradrenergic fibers with DBH-saporin increased the severity of mechanical hypersensitivity for 4 months in the ipsilateral paw after chronic constriction injury and that spinal blockade of α2-adrenergic receptors after tibial nerve transection hastened the onset of ipsilateral mechanical hypersensitivity.

Once mechanical hypersensitivity was established α2-adrenergic receptor blockade did not further increase ipsilateral hypersensitivity, suggesting that the noradrenergic system spatially restricts and temporally delays the onset of neuropathic pain but may be insufficient to completely block ipsilateral sensitization. In contrast to our results with spinal noradrenergic fiber ablation, other data suggest that supraspinally projecting noradrenergic neurons in the LC may facilitate neuropathic hypersensitivity. LC projections are topographically organized, with ventral LC and subcoeruleus neurons preferentially innervating the dorsal spinal cord and dorsal LC core neurons predominantly innervating the cortex, amygdala, and hippocampus. In accordance with this, selective activation of ventral neurons in the LC results in antinociception, whereas stimulation of more dorsal core regions of the LC results in pronociception. Therefore, the behavioral effects of ablative or pharmacological strategies to disrupt the noradrenergic system on mechanical hypersensitivity are dependent on the location of ablation and the time of testing which may partially explain previous discrepant results.
In addition to evoked responses to exogenous stimuli, we show that disrupting spinal noradrenergic innervation after surgery affects behavior potentially indicative of spontaneous pain. As such, intrathecal anti-DβH-saporin pretreatment hastened the shifting of weight away from the hind paw ipsilateral to injury although it did not affect the time course of recovery. The shorter time course of this nonevoked measure of pain compared with hypersensitivity is consistent with other studies in neuropathic pain models.\textsuperscript{43} We speculate that this shorter time course of recovery in weight bearing compared with evoked responses in pSNL rats may be due in part to the development of contralateral hypersensitivity.

Spinal glial activation likely contributes to central sensitization and mechanical hypersensitivity after surgical nerve injury.\textsuperscript{44,45} We show that a reduction in spinal norepinephrine results in long-term increases in spinal glial activation after peripheral nerve injury, similar to previous studies in our laboratory that examined earlier time points.\textsuperscript{22} Consistent with our findings, noradrenergic receptor agonists have been shown to inhibit spinal glial activation \textit{in vivo} in rodent neuropathic pain models\textsuperscript{46,47} and \textit{in vitro} in spinal microglia\textsuperscript{48} and astrocyte cultures.\textsuperscript{49} Enhanced glial activation after injury likely results from increased primary afferent release of stimulating factors (e.g., glutamate, substance P) due to a loss of presynaptic α2A inhibition\textsuperscript{50} or due to a reduced inhibitory or antiinflammatory influence of norepinephrine through direct effects on α2- or β-adrenergic receptors expressed on spinal glia.\textsuperscript{48,49,51} Future studies are needed to determine whether the enhanced glial activation in norepinephrine-depleted rats is causally related to the delayed recovery of hypersensitivity.

\textbf{Role of Descending Noradrenergic Signaling after Recovery: A New Homeostasis}

Behavioral recovery from sensory sensitization may reflect a new balance of increased inhibitory and excitatory influences. Opioid systems have been most studied in this regard, and recovery from hypersensitivity after high-dose opioid agonist exposure\textsuperscript{62} or inflammation can be reversed by naloxone although naloxone is without effect on sensitivity in normal animals.\textsuperscript{17} We observed a reinstatement of ipsilateral pain when DβH-saporin was delivered to rats that recovered from hypersensitivity after pSNL providing evidence that the spinal noradrenergic system is involved in returning withdrawal threshold to baseline after surgery. This is consistent with the observation that mechanical hypersensitivity in the ipsilateral paw is unmasked by delivery of spinal α2-adrenergic receptor antagonists to rats that fail to develop allodynia from a surgical injury.\textsuperscript{53,54} The mechanisms by which spinal noradrenergic tone is increased in a sustained manner after injury are unclear. We previously observed anatomic plasticity, with up-regulation of noradrenergic innervation (DβH-IR) within the spinal cord after chronic sciatic nerve constriction\textsuperscript{55} and complete L5/L6 spinal nerve ligation\textsuperscript{28} although others report a decrease in density of spinal DβH-positive fibers in the ipsilateral spinal cord after tibial nerve transaction.\textsuperscript{9} In addition, basal spinal norepinephrine release and spinal norepinephrine content are also increased after complete L5/L6 spinal nerve ligation injury.\textsuperscript{56} In the current study, we extend our previous observations to a less severe model of nerve injury that resolves in 5 to 10 weeks. We observed increased spinal norepinephrine content as early as 21 days until greater than 3 months after pSNL, time points that correspond to the time course of resolution of mechanical hypersensitivity. Based on the effects of postsurgery DβH-saporin, this ongoing increased noradrenergic tone may suppress the latent neuropathic hypersensitivity in the current model.

\textbf{Method Development for Studying Individual Differences Linking CPM to CPSP}

Individual differences and variability in response to CPM and nerve injury in animal models of neuropathic pain may depend on strain, species, environment, and type of surgical injury.\textsuperscript{57} We observed considerable interanimal variability in strength of CPM as well as in the rate of recovery of mechanical hypersensitivity likely due to a combination of genetic and environmental factors as we use a genetically heterogeneous outbred strain of rats. We posit that some of this variability is due to differences in the ability to engage descending noradrenergic inhibitory pathways. Interstrain and intrastrain differences in the engagement of descending inhibition have been reported after peripheral nerve injury in rats.\textsuperscript{53} Similarly, strains of mice differ in stress-induced analgesia and endogenous opioid-mediated inhibition.\textsuperscript{58–60} Thus, individual differences in biological responses to stress (induced by CPM or surgery) or differential engagement of other endogenous inhibitory circuits\textsuperscript{58} may also contribute to the observed variability.

Clinically, the association between CPM and CPSP has relied on definition of CPSP as pain being present or absent at an arbitrary time after surgery. Because recovery from pain occurs for years postoperatively,\textsuperscript{61,62} this dichotomous approach at a single time fails to accurately depict the recovery process. In the current study, we chose to define recovery from hypersensitivity in a nondichotomous manner using growth curve modeling,\textsuperscript{29} as we and others are applying to define time course of recovery from pain after surgery in humans.\textsuperscript{53,64} Collectively, these studies suggest that targeting therapies that augment descending inhibition (e.g., norepinephrine reuptake inhibitors) to patients with reduced preoperative CPM may decrease the severity and incidence of CPSP in those most at risk. Less efficient CPM in patients with chronic diabetic neuropathy predicted analgesic efficacy of duloxetine\textsuperscript{65} and tapentadol\textsuperscript{66} in recent studies; however, similar studies involving relatively pain-free patients before surgery have not been conducted.

In summary, the ability to engage descending inhibition during acute nociception correlates with time course of recovery from hypersensitivity after surgery in rodents, mirroring the correlation in humans. These data suggest that descending
noradrenergic pathways are important to acute inhibition and that their sustained activation is important to the recovery after neuropathic injury.

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Competing Interests

The authors declare no competing interests.

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