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Detection of Neuropathic Pain in a Rat Model of Peripheral Nerve Injury

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Background: **Behavioral criteria that confirm neuropathic pain in animal injury models are undefined. Therefore, the authors sought clinically relevant measures that distinguish pain behavior of rats with peripheral nerve injury from those with sham injury.**

Methods: **The authors examined mechanical and thermal sensory sensitivity, comparing responses at baseline to responses after spinal nerve ligation (SNL group), sham nerve injury (sham group), or skin incision alone (control group).**

Results: **Substantial variance was evident in all sensory tests at baseline. After surgery, tests using brush, cold, or heat stimulation showed minimal distinctions between surgical groups. Postsurgical thresholds for flexion withdrawal from mechanical stimulation with von Frey fibers were decreased bilaterally in SNL and sham groups. In contrast, the probability of a complex hyperalgesia-type response with prolonged elevation, shaking, or licking of the paw was selectively increased on the ipsilateral side in the SNL group. Nonetheless, the effect of SNL on behavior was inconsistent, regardless of the sensory test. The behavioral measure that best distinguishes between SNL and sham groups and thereby best identifies animals with successful SNL-induced neuropathic pain is increased ipsilateral postsurgical probability of a hyperalgesia-type response to noxious mechanical stimulation. Using receiver operating characteristics analysis, mechanical hyperalgesia identifies a local SNL effect in approximately 60% of animals when specificity is required to be 90% or higher.**

Conclusions: **Simple withdrawal from von Frey tactile stimulation, although frequently used, is not a valid measure of peripheral nerve injury pain in rats, whereas a complex hyperalgesic-type response is a specific neuropathy-induced behavior.**

EXPLORATION of the mechanisms producing neuropathic pain has been aided by use of rodent models of peripheral nerve injury. In such studies, evaluation of spontaneous and evoked behavior serves as indirect evidence of pain, which is conventionally defined as "an unpleasant sensory and emotional experience."[1] In addition to the inherent weakness of inferences about animal experience, there have been other important limitations to studies using animal models. A wide variety of sensory testing methods have been used, often alone rather than in combination. Because nerve injury has a nonuniform effect on different sensory modalities,[2–6] the evident effect of injury is critically dependent on the choice of test. Furthermore, the specific hallmarks necessary to document neuropathic pain in animals are undefined. For example, decreased threshold for reflex withdrawal from a tactile stimulus, although commonly used as an outcome criterion, has not been validated as an indicator of an unpleasant experience.

Spinal nerve ligation (SNL) is a popular model of peripheral nerve injury that produces incomplete denervation of the sciatic nerve sensory territory[7] through selective damage of a subset of spinal nerves forming the sciatic nerve. Because resulting behavior after injury may vary substantially between subjects,[2,6] group averages may show effects that are not reliably present in all animals. For this reason, studies of electrophysiologic and pharmacologic mechanisms require inclusion criteria that identify suitable animals with successful SNL-induced neuropathic behavior.

Therefore, we sought to clarify the modality-specific sensory effects of peripheral nerve injury by SNL. To identify patterns of change in behavior relevant to the human experience of neuropathic pain,[8] we stipulated that changes would be deemed as valid representations of animal pain only if increased responsiveness was predominantly ipsilateral to injury and if changes were more evident in fully injured animals than in those subjected to sham surgery, thus dissociating the specific neural injury effect of the model from nonspecific global changes in sensory responsiveness and distant nonneural injury effects.[9,10] We hypothesized that, despite inherent variability in sensory behavior and inconsistent expression of nerve injury effects, a subgroup of tests would distinguish those SNL animals that successfully exhibited a specific local effect representing animal neuropathic pain.

Materials and Methods

Surgery

A total of 150 male Sprague-Dawley rats from a single vendor (Charles River Laboratories Inc., Wilmington, MA) were used for these studies. Their diet (LabDiet 5001; PMI Nutrition International, St. Louis, MO) contained 23% protein derived approximately 50% from soy and contained 459 μg/g total phytoestrogens. After approval by the Animal Care and Use Committee of the Medical College of Wisconsin (Milwaukee, Wisconsin), animals weighing 160–180 g were randomly assigned to an SNL group, a sham surgery group, or a control group. Animals within a cohort that arrived at the laboratory together were divided among the surgery groups. For SNL,[11] rats were anesthetized with halothane (2–3%) in

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oxygen, the back was shaved, and the right lumbar paravertebral region was exposed. After subperiosteal removal of the sixth lumbar transverse process, both the right fifth and the sixth lumbar spinal nerves were tightly ligated with 6-0 silk suture and transected distal to the ligature. To minimize nonneural injury, no muscle was removed, muscles and intertransverse fascia were incised only at the site of the two ligations, and articular processes were not removed. The lumbar fascia was closed by 4-0 resorbable polyglactin suture, and the skin was closed with three staples, which were not removed during the study interval. Sham surgery consisted of an identical procedure except that the nerves were not ligated or sectioned after exposure. Animals in the control group had only anesthesia and a lumbar skin incision. At the end of the sensory testing series, each animal was killed by anesthetic overdose, and the nerve injury site was examined by dissecting microscope (15×).

Sensory Testing

At least 1 day after arrival to the animal care facility, animals were brought to the testing area for 4 h of familiarization with handling and the environment. Subsequently, testing sessions were performed on the day preceding surgery and on the fourth, eleventh, and eighteenth days after surgery. All testing was performed by two investigators (Q. H. and K. M.-J.), who maintained concordant technique and scoring by at least weekly joint testing sessions.

Our overall strategy involved measuring changes in sensory test responses over time and in different surgery groups. We sought to identify the testing methods for which ipsilateral change in the SNL group contrasts maximally with that in animals without ligation. Such tests can then be considered the best indicators of the SNL effect and therefore, by our criteria, selective indicators of neuropathic animal pain. Tests were chosen for study by their relevance to changes noted in clinical neuropathic pain.[8] Testing examined the plantar skin of each hind paw of unrestrained rats, including the following procedures in each case. Except for heat response, testing was performed with the animals on a ¼-in wire grid, placed individually in clear plastic enclosures (10 × 25 cm).

Heat. Animals were placed on temperature-regulated glass and exposed to a radiant heat source.[12] Three determinations of withdrawal latency for each paw were separated by 5 min.

Light Touch. An 8-mm-wide camel hair brush was stroked longitudinally along the center of the paw. The response was scored as either none or positive if the paw was removed. The test was applied three times to each paw, separated by intervals of at least 1 min.

Cold. Acetone was expelled through tubing to form a meniscus that was touched the central skin without contact of the tubing with the skin.[13] The response was scored as either none or positive if the paw was removed. Positive responses were uniformly brief (less than 10 s), and three repetitions were spaced at least 2 min apart.

von Frey Fibers. Punctate mechanical stimulation was applied by von Frey fibers (Smith and Nephew Inc., Germantown, WI). Care was taken to approach the skin slowly to standardize the force–time relation during stimulation. Contact was made for 1 s with a force just adequate to bend the fiber. Ten separate sites were tested throughout the plantar surface of the paw, four aligned in the center of the paw and three each medially and laterally,[14] avoiding the paw pads and the hairy skin. Fibers with forces of 0.57, 0.84, 1.39, 2.27, 4.03, 5.13, 6.92, 11.0, 14.2, and 24.6 g were applied in increasing order from the weakest to strongest. All regions (middle, medial, lateral) of the right and left paw were tested, in random sequence, before going on to the next stiffest fiber. A method of constant stimuli was used in which all animals were tested with the full range of fibers regardless of their responses. The withdrawal response was scored either as none or positive if the paw was removed. If there was no response, the value of 25 g was assigned as threshold. In addition, we recorded whether the motor response was a brief flinch or whether stimulation caused the rat to hold the paw in the air for a second or more or to shake, groom, lick, or chew the paw. We have termed this a *hyperalgesia-type response* because of its complexity and duration, without making any assumption regarding mechanism or threshold.

Testing with von Frey fibers was performed using fibers modified with blunt tungsten tips[14] of 100- or 200-μm diameter to standardize the contact area and to produce a more selectively nociceptive stimulus (von Frey, as quoted by Bishop[15]). For comparison with other reports, a small group of animals were tested using standard fibers with unaltered tips that vary in diameter from 0.16 to 0.54 mm for the fiber strengths used.

Needle. The point of a 22-gauge spinal anesthesia needle was applied to the center of the paw with enough force to indent the skin but not to puncture it. Responses were of two types, either a brisk simple withdrawal with immediate return of the foot to the cage floor or a hyperalgesia-type sustained elevation with licking and grooming. The response type was noted for each of three applications to each paw separated by at least 2 min.

Testing Protocol

Animals rested in the test enclosures for 30 min before testing. The type of surgery for each animal was unknown to the examiner performing the sensory testing, although there was no means of concealing postural abnormalities of the paw. The sequence of testing was as listed above except that, on a random basis, half the animals in a testing group would have heat response

determined last. Before all stimulus presentations, the cage bottom or plastic enclosure was lightly tapped to aid in producing a constant arousal state. The side of the first presentations was alternated randomly. At least 10-min intervals separated different sensory tests.

Measures and Statistics

The force generated by each fiber after any tip modification was determined on an analytic scale and used for relevant calculations. The von Frey fiber force resulting in a 50% withdrawal rate for each paw was determined in a manner similar to that of Song *et al.*[14] Briefly, the logit transformation of response probability was calculated as ln(P/(1 − P)), in which P is the probability of response to that fiber strength. For P of 0 and 1, the numbers 0.05 and 0.95 were substituted. (When the number of trials is 10, these default values are the same as (2n − 1)/2n and 1/(2n) used by Song *et al.*[14] For determinations of thresholds for regions of the foot using 3 or 4 trials, the calculated threshold differed by less than 0.5% using 0.05 and 0.95 compared with the exact method of Song *et al.*[14]) Interpolation was achieved by graphing the logit transformation against the log of the milligram force for each fiber. A linear fit of this central segment allowed calculation of the point at which the logit was 0, indicating the gram force producing 50% response. In this way, the 50% threshold was determined for the whole paw, and the data for the different regions of the paw (middle, medial, lateral) were used to calculate separate 50% thresholds for each region. For other sensory modalities, the repeat determinations on a particular day were averaged for each paw.

Main effects were tested by analysis of variance, and a repeated-measures analysis of variance model was used to test the effect of site of the paw and the effect of time. *Post hoc* assessment of within comparisons was performed conservatively using the Bonferroni test (Statistica 6.0; StatSoft, Tulsa, OK). Significance levels were set at 0.05. Graphs show means ± 95% confidence intervals.

In addition to determining the significance of differences between means, we also gauged the value of the various sensory measures by how well each revealed the difference between surgery groups. By signal detection theory,[16] the ability of a measure to discriminate between groups is proportionate to the strength of the signal, which in the current case is the difference between the means for the SNL and sham groups, and is limited by the extent of noise, or inherent variability of the behavioral measure. We calculated the discriminability index of the tests (d') as the difference of the means divided by the average SD of the sham and SNL groups.[17] The cutoff point, or critical value, of a measure is the chosen value above which the measure is considered positive and below which it is considered negative. The most desirable cutoff point is that which optimizes both sensitivity (number of SNL animals with a positive test divided by the number of SNL animals tested) and specificity (number of uninjured animals with a negative test divided by the number of uninjured animals tested). Receiver operating characteristic curves were plotted to compare sensitivity and specificity for the relevant measures at multiple cutoff values. The area under the curve for the receiver operating characteristic curves, an indicator of the overall effectiveness of the test in distinguishing the groups,[18] was calculated using the trapezoid rule, which may mildly underestimate the value derived by curve fitting through maximal likelihood estimation.[19]

Results

Nonsensory Effects of Surgery

Post mortem examination confirmed accurate section and placement of ligatures in all SNL animals. Weight gain was not affected by SNL (7.02 ± 0.22 g/day; n = 30) or sham surgery (6.94 ± 0.26 g/day; n = 29) compared with control animals (7.44 ± 0.46 g/day; n = 30). Abnormal ipsilateral paw posture was noted throughout the postsurgical period in 16% of the SNL animals, and an additional in 21% showed deformity at the first testing session only. These animals held the toes together and the paw inverted, usually avoiding contact with the floor, comparable to behavior described by Kim and Chung following SNL.[11] One sham animal showed ipsilateral deformity. No spontaneous licking of the paw or autotomy was seen. The characteristic severe motor deficit and limping ambulation seen after injury to the L4 spinal nerve[11] was not evident in any of the animals.

Tactile Withdrawal Threshold Determination

For threshold estimation using logistic transformation, a reliably linear relation between logit value and log of force was indicated by a covariance coefficient of 0.907 ± 0.006 for determinations of the entire foot using 10 applications of each fiber, 0.853 ± 0.016 for the middle foot using 4 applications, and 0.875 ± 0.013 for the medial and lateral foot determinations using 3 applications. These findings are comparable to those of Song *et al.*[14] and represent a reliable linear relation between logit value and log of force.

Baseline Sensory Findings

Before surgery, thresholds for withdrawal from von Frey fiber stimulation depended on tip diameter and stimulus location (table 1). Testing with fibers having 100-μm-diameter tips resulted in withdrawal thresholds lower than those determined using fibers with 200-μm tips or unaltered von Frey fibers. Using unaltered fibers, 8 of 12 feet tested did not achieve a 50% response using the stiffest fiber, which never occurred with the tungsten tips. Thresholds values were lower in the lateral

Table 1. Baseline von Frey Withdrawal Thresholds

Probe	No. of Rats	Entire Foot‡	Middle	Medial	Lateral
100	43	3.48 ± 0.24	4.27 ± 0.31	3.71 ± 0.32	2.44 ± 0.22*†
200	61	5.61 ± 0.27	9.57 ± 0.34	4.88 ± 0.34*	3.78 ± 0.25*†
200N	32	14.46 ± 0.69	14.35 ± 0.62	14.77 ± 0.70	13.81 ± 0.85
Variable	6	27.25 ± 4.58	27.70 ± 1.54	23.90 ± 2.11	15.34 ± 0.79*†

Baseline withdrawal threshold (in grams) to von Frey fiber stimulation before surgery, using fibers with tip diameters of 100 μm (100 probe) or 200 μm (200 probe) or unaltered tips (variable probe). With the 200N probe, 200-μm–tipped fibers were applied, avoiding the glabrous skin margin. Fibers were applied with a single touch at each of 10 sites, with thresholds calculated for the entire foot or region. Threshold was determined by interpolation of logit values (see text). Data are presented as mean ± SE.

* Significantly different from middle. † Significantly different from medial. ‡ All comparisons between probes are significantly different except 100 probe *vs.* 200 probe.

region compared with medial and middle in animals tested with either 100-μm tips or 200-μm tips. Unmodified fibers resulted in higher thresholds but still showed a relatively increased sensitivity in the lateral region of the paw. We tested additional animals with 200-μm-tipped fibers applied in a narrower pattern, such that probe contact sites were at least 1 mm within the glabrous margin for the medial and lateral determinations. Tested this way, lateral thresholds were comparable to those in the medial and middle regions of the paw.

In response to von Frey fiber application, the more complex hyperalgesia response with sustained lifting, licking, grooming, and chewing was noted too rarely at baseline to define a force threshold for this behavior. Therefore, to characterize individual animals, the probability for such a response during the application of the five fibers ranging in force from 2.27 to 11.0 g (a total of 50 touches) was averaged for each paw (table 2). The probability of a hyperalgesia-type response was greater with the smallest tipped fibers than with 200-μm tips, and no hyperalgesia responses were noted using fibers with unmodified tips. Whereas the lateral margin of the paw was the most responsive region for withdrawal response, this part of the paw was less responsive than others when measured by the probability of producing a hyperalgesia response.

A prominent feature of baseline testing was wide variability in sensory responsiveness, evident in large SEMs. Variance in the data could be apportioned between the 20 cohorts of animals that arrive as a group, typically six to nine rats, and to a component of variance within the cohorts. For the various sensory tests, between 72% and 94% of variance (calculated as between-cohort variance divided by the sum of error variance and between-cohort variance, multiplied by 100) is attributable to differences between cohorts. To examine whether this variability represents true differences in responsiveness rather than testing unreliability, the absolute difference in thresholds for withdrawal of the right and left paws of individual animals was determined as 0.48 ± 0.09 and 0.63 ± 0.16 g for 100- and 200-μm probes, respectively, representing a small fraction (14%, 11%) of the mean thresholds. This shows consistency between right and left paws despite substantial differences between cohorts.

Sensory Changes after SNL: Group Averages

Heat. Spinal nerve ligation resulted in a shortened average latency on the ipsilateral side on day 18 after injury compared with baseline, but there were no differences between ipsilateral and contralateral paws (fig. 1A). There were no changes in latency in the sham surgery and control groups.

Light Touch. There was a bilateral increase in probability of response to stroking by a brush compared with baseline in both the sham and SNL groups and an increase in control animals that reached significance on the contralateral side (fig. 1B). An increased responsiveness developed in the ipsilateral compared with the contralateral paw, but this was temporary and present in both the sham and SNL groups.

Cold. Testing with acetone showed a bilateral increase in probability of response compared with baseline in both the sham and SNL groups (fig. 1C). An increased responsiveness developed in the ipsilateral compared

Table 2. Baseline von Frey Hyperalgesia Probability

Probe	No. of Rats	Entire Foot	Middle	Medial	Lateral
100	43	6.19 ± 0.90‡	5.84 ± 1.24	9.12 ± 1.56	3.71 ± 0.76*†
200	61	1.11 ± 0.35	1.09 ± 0.31	1.54 ± 0.37	0.75 ± 0.44
200N	32	0.31 ± 0.11	0.44 ± 0.14	0.22 ± 0.09	0.22 ± 0.09
Variable	6	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Baseline probability (in percent) of a hyperalgesia-type response (see text) to von Frey fiber stimulation before surgery, using fibers with tip diameters of 100 μm (100 probe) or 200 μm (200 probe) or unaltered tips (variable probe). With the 200N probe, 200-μm–tipped fibers were applied, avoiding the glabrous skin margin. Fibers were applied with a single touch at each of 10 sites. There were no hyperalgesia responses using the unmodified von Frey fibers with variable tip diameter. Data are presented as mean ± SE.

* Significantly different from middle. † Significantly different from medial. ‡ Significantly different from all other probes.

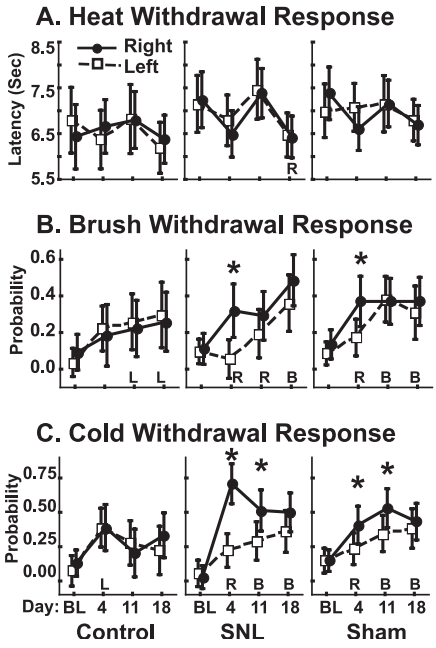

Fig. 1. Average latency of response for withdrawal from heat (*A*) and average probability of withdrawal from brush (*B*) and cold (*C*). For each test modality, the number of rats is 28 in the control group, 29 in the spinal nerve ligation (SNL) group, and 27 in the sham group. BL = baseline. * = Significant difference between ipsilateral (right) and contralateral (left); R = significant difference from baseline at that time point for right paw; L = difference from baseline for left paw; B = difference from baseline for both paws. Differences were evaluated by analysis of variance with *post hoc* Bonferroni test. *Error bars* show 95% confidence intervals.

with the contralateral paw, but this was temporary and present in both the sham and SNL groups.

von Frey Fibers.

Withdrawal Response. Spinal nerve ligation effects (fig. 2) were monitored with von Frey fibers having 100-μm tips, applying the probes up to the margins of the glabrous skin. (Similar results were found with 200-μm tips; data not shown.) For the entire paw and individual regions, withdrawal threshold decreased in all surgery groups bilaterally. Only on day 11 in the SNL group was the threshold less on the ipsilateral side compared with the contralateral side.

Because of extensive variability in the individual response patterns, few animals showed response curves over time that resembled the group averages (fig. 3). Comparing ipsilateral to contralateral changes between successive testing sessions revealed a strong tendency for these to move in parallel regardless of surgery group (fig. 4), which indicates a shift in general excitability superimposed on injury effects. Therefore, to improve resolution of surgical effects, the responses for the three postsurgical days were averaged for each paw of each rat. To score the ipsilateral threshold decrease, the degree to which the change in the right exceeded the change in the left ($\Delta R - \Delta L$) was calculated (details in legend of table 3). This score was generated for the entire paw and for the middle region separately, but neither showed significant differences between surgery groups. Asymmetry in postsurgical von Frey withdrawal thresholds was also evaluated without reference to baseline values (psR–psL; details in legend of table 3). This score was significantly different between sham and SNL groups only for the whole paw.

Hyperalgesia-type Response. The probability of a hyperalgesia response to von Frey stimulation with 100-μm-fiber tips (fig. 2) was substantially increased uniquely in the ipsilateral paw of the SNL group. The high variability was addressed by averaging the three postsurgical test days (table 4). Both the $\Delta R - \Delta L$ and psR–psL scores (defined in the legend of table 4) in the SNL group were distinct from the sham and control groups. The differences were especially marked in the lateral region of the paw.

Needle. Probability of a hyperalgesia response to needle stimulation showed a clear difference between ipsilateral and contralateral sides in the SNL group alone (fig. 5) and a significant increase in the ipsilateral probability compared with baseline. Both the $\Delta R - \Delta L$ and the psR–psL for probability of response to needle stimulation were markedly greater in the SNL group than the sham or control groups (table 4).

Identification of Individual SNL Successes

The difference in group means of the $\Delta R - \Delta L$ and psR–psL scores does not assure that they can usefully distinguish SNL animals from sham animals, which is our proxy for identifying neuropathic pain. Therefore, these scores were evaluated on the basis of their discriminability index d', which was 1.0 or greater for a subgroup of tests (tables 3 and 4), particularly those evaluating a hyperalgesic-type response to tactile stimulation. Receiver operating characteristics curve analysis (fig. 6) showed areas under the curve greater than 0.80 only for measures of hyperalgesia-type response probability (tables 3 and 4). Values of receiver operating characteristics curve areas correlated significantly with d' ($R^2 = 0.84$, $P < 0.05$). Combinations of sensory measures[20] did not produce a combined score with an area under the receiver operating characteristics curve greater than the individual scores alone (data not shown).

Discussion

Inconsistency and ambiguity in behavioral outcomes after nerve injury may limit the relevance of these models for examination of neuropathic pain. Our goal was to develop the means of identifying animals in which nerve injury had successfully produced animal pain despite these impediments. The heuristic we used is based on explicit assumptions and criteria. Specifically, the sensory modalities we examined were chosen because neu-

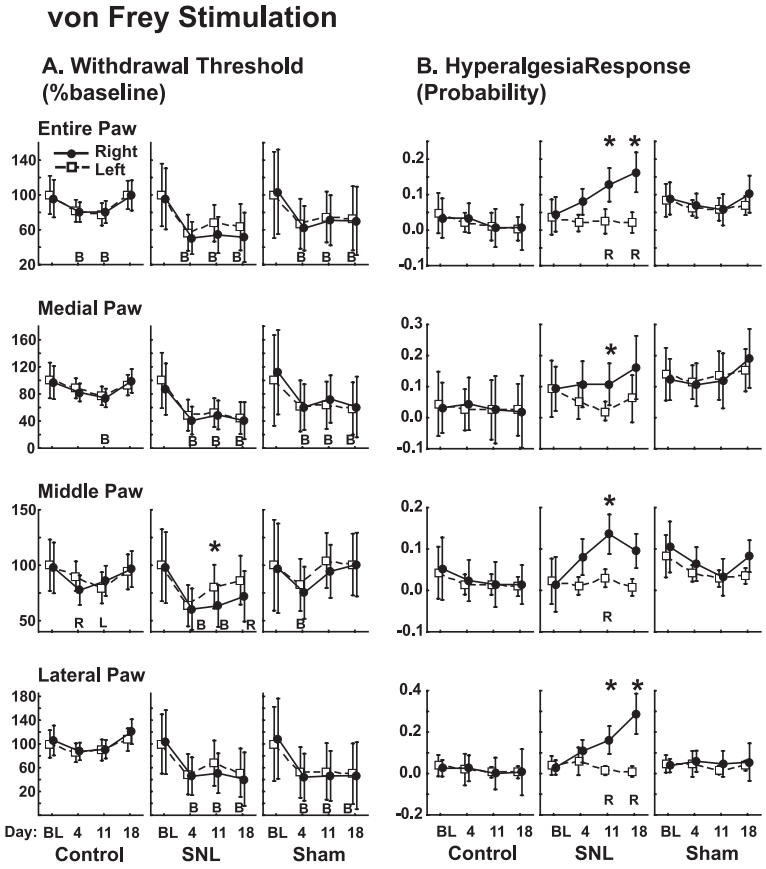

Fig. 2. Responses over time to von Frey fiber stimulus using 100-μm tips in different surgical groups. (*A*) Threshold for simple withdrawal response for the entire foot and individual regions. Threshold is normalized to 100% baseline (BL) left paw threshold. (*B*) Probability of a hyperalgesia-type response for the entire foot and individual regions. Ordinate is the ratio of hyperalgesia responses to total fiber applications (50 touches of fibers with forces of 2.27–11.0 g; see text). For both tests and each paw region, the number of rats is 10 in the control group, 16 in the spinal nerve ligation (SNL) group, and 15 in the sham group. * = Significant difference between ipsilateral (right) and contralateral (left); R = significant difference from baseline at that time point for right paw; L = difference from baseline for left paw; B = difference from baseline for both paws. Differences were evaluated by analysis of variance with *post hoc* Bonferroni test. *Error bars* show 95% confidence intervals.

ropathic pain patients present with hypersensitivity to mild and intense mechanical and thermal stimuli.[8] We used tests that are established in animal experimentation and focus on cutaneous sensation and are thus readily accessible to direct stimulation. The measures made with these tests were used to generate scores that compare between sides. Although mirror pain may occasionally accompany clinical neuropathic pain, injury-induced neuropathic pain is rarely symmetric. Also, because injury unrelated to nerves may generate distant cutaneous tactile hypersensitivity, as shown in the bilateral responses to sham surgery in this study and in the observations of others,[9,10] asymmetry is a necessary criterion to distinguish behavior specifically attributable to peripheral mononeuropathy. The alterative approach of considering any increased responsiveness, including contralateral to the injury, as evidence of neuropathic pain would attribute neuropathic pain to animals with hypersensitivity after nonneural injury by sham surgery. Finally, we reasoned that the most desirable scores for identifying neuropathic pain after nerve ligation are those that best discern between animals in the SNL and sham surgery groups. This functional definition is necessary because of the unavoidable lack of an accepted standard for neuropathic animal pain, and it accurately reflects the implicit standard. Nonetheless, it is inevitable that complete validation of behavior representing pain in animals is impossible, because the experience of the animals can never be determined.

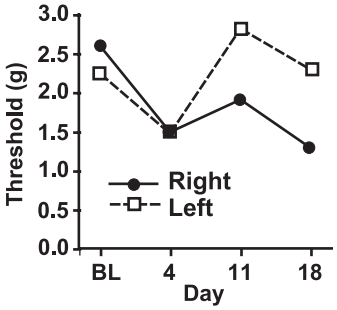

Fig. 3. Example of time course for withdrawal thresholds from von Frey stimulation with 100-μm tips for a spinal nerve ligation rat. For most animals, such as the rat shown, there is bilateral variability indicated as a tendency for parallel movement of thresholds in the right and left paws, superimposed on a unilateral injury effect represented as the difference between paws at each time point. BL = baseline.

Several principal observations emerge from the data of this study. Baseline response to paw stimulation is highly variable regardless of the sensory modality of the stimulus. Substantial nonspecific increase in responsiveness is evident after sham surgery and contralateral to nerve injury by SNL. However, a complex guarding and grooming response resembling human hyperalgesia is selectively increased on the ipsilateral side in the SNL group. Nonetheless, there is inconsistent success in producing behavioral changes by SNL. A successful preparation can be selected with adequate sensitivity and specificity by

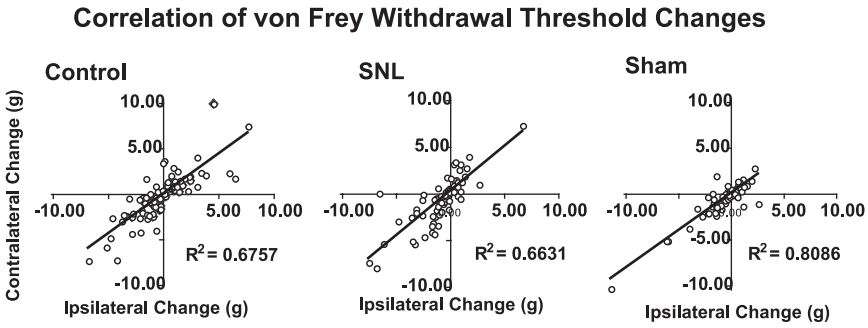

Fig. 4. Side-to-side comparison of change in withdrawal response threshold for von Frey stimulation of the whole paw. Each *data point* represents the incremental change for the right (ipsilateral) and left paw between two adjacent time points. A strong tendency for parallel shifts indicates an overriding bilateral influence on responsiveness. The trend line and squared correlation coefficient are shown in each case. SNL = spinal nerve ligation.

time-averaged measures of hyperalgesia responses to mechanical stimulation.

Variability of Behavioral Measures

The skin has nonuniform sensitivity, with low-threshold sensory "spots" separated by zones of diminished tactile sensitivity.[15,21,22] In addition, response thresholds differ between subjects.[23–25] To address these sources of variability, tactile stimuli were distributed to multiple sites across the paw, which improves the sample size for measurement of an inconsistent event and produces an averaged result for an area with a nonuniform distribution of sensitivity. The method we used also exposes all animals to the same stimulus set for consistency, and the interpolation technique uses data from all von Frey fibers (100 applications/foot) to arrive at the 50% response threshold. Fiber tips were standardized because the wide span of tip diameters of unaltered von Frey fibers distributes the highest force to an area 100-fold larger than the weakest fiber, substantially altering the nature of the stimulus.[15,26] Lower thresholds using 100-μm tips compared with 200-μm and unaltered tips is consistent with the inverse relation between probe size and discharge rates for myelinated and nonmyelinated fibers innervating the rat paw.[27]

We noted a lower threshold for lateral and medial portions of the plantar skin that was not evident when stimulation of the glabrous skin margin was avoided, where hair and field units are activated by forces as low as 6 mg.[28] In contrast, the dominant mechanosensory units of the rat planter skin, excluding the toes and pads, are type II slow-adapting receptors with individual cell thresholds of approximately 0.6–0.8 g.

There is considerable variability in baseline testing of all sensory modalities despite standardizing the source of animals, laboratory conditions, and testing techniques. The close match of right and left paws assures us that this is not due solely to random variation in responses or posture of the paw,[29] but rather is due to interindividual differences in reactivity. Much of this can be attributed to variance between cohorts of animals delivered to the laboratory as a group. Although all rats were a single strain from a single vendor, we learned after data collection that they originated from five colonies in three different states. Migration of breeder rats between colonies limits genetic divergence,[30] but minor genetic differences between colonies may still develop that alter sensory behavior,[31–33] and early experience before or during shipping may change cutaneous thresholds.[34,35]

Behavioral Response to Nerve Injury

The effects of nerve injury are not uniform between different sensory modalities. We found that responses to cold, heat, and low-intensity mechanical stimulation did not reliably differ between SNL and sham animals or between the injured and contralateral sides after SNL.

Table 3. Injury Effects on von Frey Withdrawal Response

		Entire Foot		Middle Foot Region	
Group	No. of Rats	ΔR–ΔL, %	psR–psL, %	ΔR–ΔL, %	psR–psL, %
Control	10	3.6 ± 3.7	0.2 ± 2.4	2.6 ± 7.6	−1.1 ± 3.3
Sham	15	−5.4 ± 2.1	−5.4 ± 1.5	−2.8 ± 4.2	−4.5 ± 4.2
SNL	16	−7.8 ± 4.5	−14.9 ± 3.3*†	−8.9 ± 6.7	−13.8 ± 4.3
d′		0.18	1.00	0.28	0.56
ROC (AUC)		0.58	0.79	0.59	0.61

Postsurgical changes in threshold for withdrawal from von Frey fibers with 100-μm tips for the entire foot and for the middle region alone. The score ΔR–ΔL represents the injury effect on the right side compared with the left, as a percent, and was calculated as 100 × [(average right postsurgical threshold) − (presurgical right baseline threshold)]/(presurgical right baseline threshold) minus the same number for the left. The score psR–psL represents postsurgical asymmetry and was calculated as 100 × [(right average postsurgical threshold) − (left average postsurgical threshold)]/(left average postsurgical threshold). Data are presented as mean ± SE.

* Significantly different from control. † Significantly different from sham.

d′ = difference between sham and spinal nerve ligation group means divided by the average SD of the groups; ROC (AUC) = area under the curve for the receiver operating characteristic curve; SNL = spinal nerve ligation.

Table 4. Injury Effects on Hyperalgesia Probability

	von Frey Stimuli				Needle Stimuli		
		Entire Foot		Lateral Region			
Group	No. of Rats	ΔR–ΔL, %	psR–psL, %	ΔR–ΔL, %	psR–psL, %	No. of Rats	ΔR–ΔL, %	psR–psL, %
Control	10	2.0 ± 0.9	0.6 ± 0.4	1.7 ± 2.4	0.4 ± 0.5	20	−4.4 ± 3.5	−1.1 ± 1.1
Sham	15	0.8 ± 1.3	1.3 ± 1.1	2.8 ± 2.8	2.0 ± 2.1	27	−2.1 ± 5.0	1.6 ± 2.5
SNL	16	11.5 ± 2.3*†	10.9 ± 1.7*†	16.1 ± 3.9*†	15.7 ± 3.4*†	28	20.0 ± 5.8*†	19.8 ± 4.3*†
d′		1.51	1.71	1.00	1.26		0.78	1.02
ROC (AUC)		0.87	0.89	0.73	0.86		0.74	0.81

Postsurgical changes in probability of hyperalgesia response to stimulation by von Frey fibers with 100-μm tips or by needle. The score ΔR–ΔL represents the injury effect on the right side compared with the left as a percent, and was calculated as 100 × [(average right postsurgical P) − (right baseline P)] − 100 × [(average left postsurgical P) − (left baseline P)], where P is the probability of a hyperalgesic response. The score psR–psL represents postsurgical asymmetry and is calculated as 100 × [(average right postsurgical P) − (average left postsurgical P)]. Data are presented as mean ± SE.

* Significantly different from control. † Significantly different from sham.

d′ = difference between sham and spinal nerve ligation means divided by the average SD of the groups; ROC (AUC) = area under the curve for the receiver operating characteristic curve; SNL = spinal nerve ligation.

The original description of SNL showed decreased response latency to radiant heat,[11] but other reports have shown no effect of SNL on heat-induced response[6] or even demonstrate hypoalgesia to radiant heat after SNL.[2] Previous studies examining cooling by acetone have shown sustained sensitivity after SNL,[6,13] unlike the temporary effect we observed, and the effects of sham surgery were not examined in these reports. In the current study, injury effects are more clearly evident in high-intensity mechanical testing with von Frey fibers and needle touch, but interpretation is complicated by three confounding factors, namely (1) general fluctuation in sensory responses, (2) altered behavior contralateral to the injury and after sham surgery, and (3) inconsistency of changes in the SNL group. These are considered in turn.

Fluctuation in Sensory Measures over Time. The sensory responses during the three postsurgical testing sessions for individual rats are not stable (fig. 3). Instead, there are typically parallel shifts in ipsilateral and contralateral sensory measures, as revealed in a strong correlation between the ipsilateral and contralateral sides for changes between tests (fig. 4). This is probably due to uncontrolled and potent influences on responsiveness, such as distraction and level of arousal and attentiveness,[36,37] which we addressed through averaging postsurgical responses over 3 separate days of testing and by comparing measures to the contralateral side. For most of behavioral tests that we examined, referencing changes to baseline values (ΔR–ΔL score) unexpectedly produced a lower d' and receiver operating characteristics curve area than comparing only postsurgical asymmetry (psR–psL score), perhaps because of the variability contributed by a single baseline determination. This indicates that averaging multiple presurgical testing sessions may be beneficial. However, our data (not shown) and others'[33] demonstrate that baseline sensory level

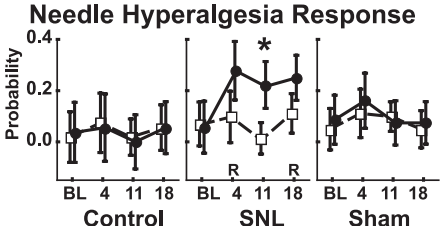

Fig. 5. Average probability for a hyperalgesia-type response (see text) from needle stimulation. The number of rats is 28 in the control group, 29 in the spinal nerve ligation (SNL) group, and 27 in the sham group. BL = baseline. * = Significant difference between ipsilateral (right) and contralateral (left); R = significant difference from baseline at that time point for right paw. Differences were evaluated by analysis of variance with *post hoc* Bonferroni test. *Error bars* show 95% confidence intervals.

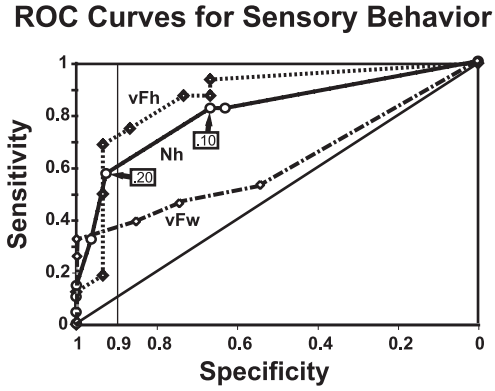

Fig. 6. Receiver operating characteristic (ROC) curves showing the ability of different methods of sensory behavior analysis to distinguish spinal nerve ligation from sham animals. Nh = psR–psL score for hyperalgesia response to needle (area under the curve, 0.81); vFh = the ΔR–ΔL score of hyperalgesia response from von Frey probe stimulation of the whole paw (area under the curve, 0.87); vFw = the ΔR–ΔL score of withdrawal response from von Frey stimulation of the whole paw (area under the curve, 0.58). The *45° straight diagonal line* represents a test with random outcomes and no ability to discriminate between groups. A *vertical line* marks 90% specificity (10% false-positive rate). *Individual data points* mark the specificity and sensitivity at a particular critical value of the score. For needle hyperalgesia response, the two points for psR–psL scores of 0.10 and 0.20 are indicated.

does not influence development of nerve injury effects in rats.

Contralateral and Sham Effects. A significant decrease in withdrawal threshold was observed with von Frey testing of the side opposite to the SNL injury. Although this agrees with various studies of peripheral nerve injury,[11,13,32,38–40] a strictly unilateral effect has been reported by others.[4,6,41] The relative contributions of various central and peripheral mechanisms to producing contralateral changes have not been resolved.[42–45] We also noted sham effects comparable to previous reports after nerve exposure[46,47] or distant nonneural injury.[10] In contrast, some studies have not observed changes in tactile sensitivity after sham surgery,[11,41] whereas other reports have not examined thresholds in sham SNL animals.[6,11,13] Generalized increased sensory responsiveness represented by contralateral and sham changes is usually less intense and less prolonged than direct SNL effects but precludes identification of neuropathic sensory consequences.

Inconsistency of SNL Effect. The effect of SNL injury on ipsilateral sensory behavior is variable. Most studies report only the average response level, but inconsistency of behavior after peripheral nerve injury has been noted by others.[2,6,48,49] We used strategies to optimize the identification of abnormal sensory responsiveness. First, we used multiple sensory modalities to enhance the opportunity for observing injury changes, but only mechanical stimuli showed significant ipsilateral/contralateral differences isolated to the SNL group. Second, because the contribution of intact L4 fibers *versus* axotomized L5 fibers is not established,[50] we separately examined the maximally denervated lateral region of the paw, the partially denervated central region, and the medial portion with minimal direct disruption of innervation. Unlike Li *et al.*,[51] our data show no consistent pattern of anatomical differences in response to injury. Why some animals show behavioral change after peripheral nerve injury and others do not is unexplained. There is clearly anatomical variability in neural pathways and peripheral nerve distribution, including inconsistent contributions by the L4 and L5 dorsal root ganglion to the sciatic nerve.[52] Furthermore, the extent of tissue damage adjacent to the injury may vary.

Overall, we recorded more modest effects of SNL than in some other reports. We saw no sudden spontaneous licking events, and abnormal posture of the ipsilateral foot was evident in only a minority of rats, even though these events are reported as typical findings in earlier descriptions.[11,53] There is a growing recognition that it is impossible to entirely control important environmental factors in animal sensory testing[54] and that findings may differ between laboratories despite intense efforts at standardization.[55] However, we believe the greatest influence on SNL effect is surgical technique. Our autopsy-controlled method was designed to minimize unintended damage, and we did not observe the characteristic motor behavior that follows L4 spinal nerve injury.[11] Ironically, our comparatively low rate in generating neuropathic behavior may be due to avoidance of L4 injury, because a recent report shows that behavioral change after SNL is proportionate to L4 damage.[56]

The extent of behavioral changes after peripheral nerve injury is highly sensitive to genetic influences,[31–33,57] which may contribute to differences between reports. Even animals of the same strain but from different vendors show dissimilar patterns of abnormal behavior after peripheral nerve injury[32,57] and even reveal contrasting anatomy and function of descending pathways regulating nociception.[58–60] Diet also strongly modulates the generation of neuropathic pain after sciatic injury, because certain levels of soy intake are required for behavioral shift from injury.[61] The need for a highly specific genetic background and carefully chosen environmental conditions might explain variations in findings, but it also raises the fundamental question of the general relevance of rodent peripheral nerve injury models. In humans, elective section of a healthy spinal nerve is an accepted component of surgical reinnervation of a damaged contralateral brachial plexus,[62] leading to only rare (one subject of five), delayed and transient hypersensitivity to mechanical and cooling stimuli.[63] Therefore, it is not clear that amplified sensory responsiveness in all subjects is an expected or desirable feature of an animal model that seeks to duplicate the human pathophysiology of peripheral nerve injury pain.

Identification of Individual Rats with Neuropathic Animal Pain

For the sake of selecting appropriate subjects for mechanistic study, it is necessary to discriminate between experimental subjects that have satisfactorily developed pain and those with an incomplete result. Other than the postsurgical asymmetry in withdrawal response to von Frey fibers, the most discerning tests measured postsurgical probability of hyperalgesia-type responses. It is a matter of judgment where to specify the critical value that establishes the boundary between values accepted as indicating neuropathic pain and those inadequate to do so, because there is an inevitably reciprocal relation between sensitivity and specificity (fig. 6). For the needle psR–psL score, the choice of 0.20 (20% hyperalgesia-type responses) as the critical value produces a sensitivity (probability that SNL rats will have a positive test result) of 57% and a specificity (probability that a sham will have a negative test result) of 93%, *i.e.*, a false positive rate of 7%. Relaxing this to a critical value of 0.10 increases sensitivity to 82% but decreases specificity to 67%. For most circumstances, such as the use of behavioral testing as an entry criterion for further mech-

anistic study, it is desirable to choose a conservative value that keeps the specificity above 90%.

Importance of the Hyperalgesia-type Response

We found that the most reliable measures for discriminating between sham and SNL injury involved a complex integrated reaction of lifting and grooming of the paw in response to mechanical stimulation. This hyperalgesia response incorporates organized unlearned behavior that indicates a sustained aversive sensory event similar to painful aftersensations reported by patients with neuropathic pain.[8] In our study, findings using this measure were similar whether the stimulus was provided by modified von Frey probes or by needle contact. Mechanical hyperalgesia is a robust test of peripheral neuropathy–induced behavior change that persists in the context of a variety of diets, whereas tactile withdrawal threshold lacks this stability.[61] After infraorbital nerve constriction, a sustained complex response is selective for the territory of the injured nerve, unlike simple withdrawal.[64] Therefore, in our study and others, a hyperalgesia-type response to clearly noxious mechanical stimulation uniquely identifies the specific pain-related behavioral effects of peripheral nerve injury.

Is the Tactile Withdrawal Response Relevant to Neuropathic Pain?

The threshold for simple withdrawal from stimulation with von Frey fibers has been widely adopted for gauging animal pain after peripheral nerve injury. However, our data indicate this type of response to mechanical stimulation is affected bilaterally and in all surgery groups and is inconsistently altered by SNL. In clinical neuropathic pain, tactile detection threshold for von Frey fibers is increased rather than decreased, whereas the response to suprathreshold mechanical stimulus is intensified.[65,66] Furthermore, intravenous opioid analgesia has no effect on von Frey perception but decreases suprathreshold mechanical hyperalgesia.[65] Therefore, unlike response to a clearly nociceptive mechanical stimulus, von Frey detection is not a relevant clinical test to distinguish neuropathic pain. Tactile withdrawal determined at threshold provides only doubtful insight regarding a fully nociceptive stimulus and may be irrelevant as an analog of clinical pain other than that which is barely perceptible.[67] The uncertain relevance of tactile withdrawal threshold determination as a test of neuropathic pain is also suggested by its failure to associate across genetically different strains with any other assays of animal pain,[68] its particular sensitivity to distant nonneural injury,[10] and its unique dependence on intact spinal cord dorsal columns,[69] a pathway predominantly serving discriminatory sensation.

A primary assumption in monitoring animal pain is that stimuli used to provoke the measured behavior are unpleasant.[70] This condition is not clearly met for tactile withdrawal, because the segmental flexion reflex underlying touch-induced withdrawal[71] persists despite decerebration, spinal cord injury, or general anesthesia,[72–74] which eliminate painful experience. Therefore, a flexion reflex alone is not adequate to establish the presence of pain. Importantly, the reflex in humans is triggered at stimulus intensities significantly below the threshold for producing pain,[75] and changes in flexion reflex do not correspond to changes in pain.[76,77] Rather than representing pain, an alternative interpretation of tactile withdrawal testing is that this form of stimulation produces sensations in the form of itch or tickle, which can be profoundly motivating without being painful.[78] Even in the uninjured state, gentle touch of the glabrous skin is the optimal stimulus for causing nonpainful aftersensations in human subjects and for producing sustained afterdischarge in the subset of dorsal horn neurons capable of doing so.[79] It is therefore possible that the many studies using flexion withdrawal from an innocuous plantar tactile stimulus as the principal measured response after nerve injury could be recast as studies examining tickle.

Overall, we believe that there is substantial doubt about the suitability of the tactile withdrawal response as a surrogate indicator of pain in animals and that evaluation of neuropathic animal pain should include examination of complex integrated behaviors, such as the hyperalgesia-type response to high-intensity mechanical stimulation. This method may be particularly appropriate for testing after SNL, because of the instability of that model across genetic and environmental domains.

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