



Enhanced formalin nociceptive responses following L5 nerve ligation in the rat reveals neuropathy-induced inflammatory hyperalgesia

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Abstract

The development of mechanical and thermal hypersensitivity following peripheral nerve injury is well known and a great deal of research has been directed towards understanding the mechanisms underlying these phenomena. However, there has been very little research examining if hypersensitivity to an inflammatory condition following nerve injury also develops. Therefore, the purpose of the present study was to determine if hypersensitivity to an inflammatory condition produced in the formalin test develops following ligation of the L5 spinal nerve. Male Sprague–Dawley rats received tight ligation of the L5 spinal nerve or were given sham surgery. Following a 14-day recovery period, the threshold to produce a withdrawal response to a mechanical stimulus was measured using von Frey monofilaments and then formalin behavioral responses were measured. Compared to sham animals, L5 ligated animals exhibited significantly lower mechanical paw withdrawal thresholds as well as elevated and prolonged nociceptive responses during the second phase (20–60 min) of the formalin test. These results reveal enhanced inflammatory nociceptive processes following peripheral nerve damage and might provide a useful approach to study underlying neural mechanisms associated with clinical neuropathic pain syndromes. © 2001 International Association for the Study of Pain. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Several experimental models, such as loose and partial ligation of the sciatic nerve (Bennett and Xie, 1988; Seltzer et al., 1990) and tight ligation of the L5 or L5/L6 spinal nerve (Kim and Chung, 1992) have been developed to explore the mechanisms underlying neuropathic pain. In these tests, the common behavioral paradigms used to infer a neuropathic condition are designed to examine altered sensitivity to mechanical and thermal stimuli. Enhanced responses, as defined by a decrease in mechanical withdrawal threshold or decrease in latency to withdrawal from a thermal stimulus, are thought to reflect the clinical conditions of allodynia (enhanced responding to a normally innocuous stimulus) and hyperalgesia (enhanced responding to a normally noxious stimulus). The mechanism by which peripheral nerve damage results in abnormal responding remains largely unknown (Gold, 2000) but might result from central sensitization to activity originating in injured afferents (Devor and Seltzer, 1994; Han et al., 2000; Liu et al., 2000) or ectopic discharge in intact C-fibers that inner-

vate skin partially denervated by ligation (Ali et al., 1999; Li et al., 2000).

Although a great deal is known about the development of mechanical and thermal sensitivity following peripheral nerve injury, there is a relative paucity of experiments that have examined if enhanced responses during chemogenic or inflammatory conditions can also develop following peripheral nerve damage (Bennett and Xie, 1988; Ceseña and Calcutt, 1999). Indeed, inflammatory conditions might be a significant clinical problem in patients who also have developed a neuropathic pain condition. One common experimental condition to explore inflammatory pain responses is the formalin test (Dubuisson and Dennis, 1977). The test involves a subcutaneous injection of dilute formaldehyde into the plantar surface of the hindpaw that causes a characteristic pattern of behavioral responses such as elevation and licking of the injected paw. There is an initial period of responding during the first 5–10 min of the test that is followed by a 5–10-min period of diminished responses that gradually return and continue for an additional 20–40 min. The second period of responding is thought to reflect an inflammatory process (Hunskar et al., 1986), signaled by peripheral mechanically insensitive afferents (MIAs) and C-fibers (Puig and Sorkin, 1995).

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Combining the methods of L5 spinal nerve ligation to model neuropathic pain and the formalin test to model inflammatory pain, the purpose of the present experiment was to determine if an increase in inflammatory responses developed following the onset of a neuropathic condition.

2. Methods

Eighteen male Sprague–Dawley rats, weighing 180–300 g (University of Texas at Arlington vivarium) were housed two to three per cage approximately 10 days prior to the beginning of the study. Animals had free access to food and water during the entire experiment and were maintained on a 12:12, light:dark schedule with the lights on at 09:00 h. The animal colony was maintained at 21°C and 60% humidity. All housing conditions and experimental procedures were approved by the University of Texas at Arlington Animal Care and Use Committee.

Experimental pain conditions were induced using 1% formalin in animals receiving ligation of the L5 spinal nerve ($n = 9$) or sham surgery ($n = 9$). Animals were anesthetized with halothane in 100% O₂ (3% induction, 2% maintenance) and nerve injury was produced by tight ligation of the L5 spinal nerve using methods previously described (Kim and Chung, 1992; LaBuda and Fuchs, 2000). Briefly, animals were placed in the prone position to access the left L4–L6 spinal nerves. Under magnification, approximately a third of the L6 transverse process was removed. The L5 spinal nerve was identified and carefully dissected free from the adjacent L4 spinal nerve and then tightly ligated using 6-0 silk sutures. The wound was treated with an antiseptic solution and was closed with wound clips. Additional animals served as sham surgery animals that were treated in an identical manner without exposure of the spinal nerves. Two weeks after nerve ligation, an inflammatory condition was induced with a 0.05-ml injection of 1% formalin solution into the plantar surface of the left hindpaw (Dubuisson and Dennis, 1977).

Immediately prior to the formalin injection, animals were tested to determine the force of a mechanical stimulus to produce a paw withdrawal response. Animals were placed within a Plexiglas chamber (20 × 10.5 × 40.5 cm³) and allowed a 15-min habituation period. The chamber was positioned on top of a mesh screen so that the mechanical stimuli could be administered to the plantar surface of both hindpaws. Mechanical threshold measurements for each hindpaw were obtained using the up/down method with eight von Frey monofilaments (5, 7, 13, 26, 43, 64, 106, and 202 mN) (Dixon, 1980). Each trial started with a von Frey force of 13 mN delivered to the right hindpaw for approximately 1 s, and then the left hindpaw. If there was no withdrawal response, the next higher force was delivered. If there was a response, the next lower force was delivered. This procedure was performed until no response was made at the highest force (202 mN) or until four stimuli

were administered following the initial response. The 50% paw withdrawal threshold for each paw was calculated using the following formula: $[X_{th}]_{\log} = [vFr]_{\log} + ky$ where $[vFr]$ is the force of the last von Frey used, $k = 0.2268$ which is the average interval (in log units) between the von Frey monofilaments, and y is a value that depends upon the pattern of withdrawal responses (Dixon, 1980). If an animal did not respond to the highest von Frey hair (202 mN), then $y = 1.00$ and the 50% mechanical paw withdrawal response for that paw was calculated according to the formula to be 341 mN. Mechanical paw withdrawal threshold testing was performed three times and the 50% withdrawal values were averaged over the three trials to determine the mechanical paw withdrawal threshold for the right and left paws for each animal.

Following mechanical paw withdrawal threshold testing, animals were tested in the formalin test (Dubuisson and Dennis, 1977). Briefly, animals were placed in a 30 × 30 × 30 cm³ Plexiglas chamber and allowed to habituate for at least 20 min. A mirror was placed below the Plexiglas chamber at a 45° angle to allow for easy viewing of behavioral responses. Subjects were administered a 0.05-ml injection of 1% formalin solution into the plantar surface of the left hindpaw. The left hindpaw was chosen since this is the paw that was afflicted by tight ligation of the L5 spinal nerve. Behavioral testing began immediately after the formalin injection and lasted for 60 min. The amount of time that each animal spent displaying one of the three behaviors was continuously recorded using a method similar to Dubuisson and Dennis (1977). Behavior was recorded as a '2' if the rat licked or bit the injected paw, as a '1' if the rat elevated the paw from the floor, and as a '0' if any part of the paw other than the tips of the digits was in contact with any surface of the box. A weighted formalin pain score for each animal at the 12 5-min test intervals was calculated using the following formula: $\text{Pain Score} = [0(\text{time spent in s with the paw down}) + 1(\text{time spent in s with inflamed paw elevated}) + 2(\text{time spent in s licking inflamed paw})]/300$ s. Therefore, a weighted formalin pain score of zero reflected an animal that spent the duration of the 5-min period with the paw down and a score of 2 indicated that the entire duration of the 5-min period was spent licking the inflamed paw.

The data was analyzed using Statistica 5.0 for Windows. Mechanical paw withdrawal threshold was analyzed using the Wilcoxon test to assess the differences between the left ligated hindpaw and the right non-ligated hindpaw for the sham surgery group and the group that had ligation of the L5 spinal nerve. The analysis of group differences of weighted formalin pain score, paw elevation, and paw licking utilized a repeated measure ANOVA with group (two levels) as the between-subjects variable, and test time period (12 levels) as the within-subject factor. Group differences at each 5-min test time period was subsequently analyzed using post-hoc comparisons (protected *t*-test). The relationship between mechanical paw withdrawal threshold and weighted forma-

lin pain score, duration of paw elevation, or duration of paw licking during the second period of the formalin test was analyzed by calculating a Pearson correlation coefficient. For each animal that had L5 ligation, the left–right hindpaw mechanical paw withdrawal threshold value was calculated and compared to the average value of each measure (i.e., weighted pain score, paw elevation, paw licking) during the 20–60-min period (phase 2) of the formalin test. The alpha level for statistical significance was set at 0.05.

3. Results

Analysis of mechanical paw withdrawal threshold for the sham surgery group revealed no significant difference between the left and right hindpaws ($P > 0.85$). However, as expected, the mechanical paw withdrawal threshold of the left ligated hindpaw was significantly lower than the withdrawal threshold of the right non-ligated hindpaw in animals that had ligation of the left L5 spinal nerve ($P < 0.01$, Fig. 1).

Formalin pain responses in both the sham surgery and L5 ligated groups followed the characteristic biphasic pattern. In both groups, there was an initial period of responding that was followed by a period of time of decreased behavioral responding. At 15–20 min following the formalin injection, there was a gradual return of responses that was maximal at 30–35 min and then gradually decreased during the remainder of the 60-min test period. The overall analysis of formalin pain responses revealed a significant main effect for

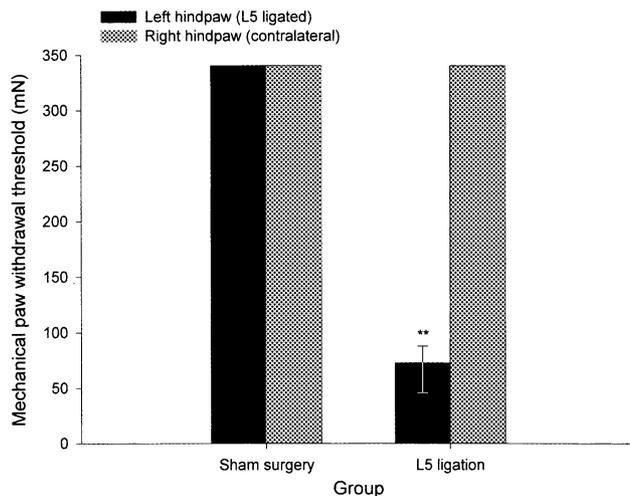


Fig. 1. Median (± 25 th/75th quartile) mechanical withdrawal threshold of the right and left hindpaws at 14 days post sham surgery or L5 nerve ligation. Measurements were made immediately prior to the formalin test. Enhanced paw withdrawal to mechanical stimulation is represented by a lower force value. Left and right hindpaw withdrawal thresholds for the sham surgery group ($n = 9$) were virtually identical. L5 ligated animals ($n = 9$) demonstrated enhanced responding to mechanical stimulation as revealed by a large decrease in withdrawal threshold of the ligated left hindpaw compared to the right hindpaw. ** $P < 0.01$ vs. contralateral hindpaw withdrawal threshold.

group ($P < 0.001$). Additional analysis at each 5-min test period revealed a lack of significant difference in responses between the sham surgery and L5 ligated groups during the first phase of responses (0–5 min, $P > 0.07$). However, additional analysis revealed that the L5 ligated group had increased formalin pain responses at virtually every time during the remainder of the test period (Fig. 2). The most obvious effect of L5 ligation on formalin pain responses was during the later portion of the test (20–60 min). Although the sham surgery group was almost finished responding to the formalin injection at 60 min, the L5 ligation group was still responding at a level that was similar to the overall 60-min average of responding demonstrated by the sham surgery group.

To further characterize the alteration in formalin pain responses following L5 ligation, paw elevation and paw licking behaviors were examined separately. As seen in Fig. 3, there was a significant increase in the amount of elevation and licking of the paw in L5 ligated animals during the second phase of the formalin test (20–60 min). There is a strong trend towards increased licking, but not elevation, during the first phase of the formalin test (0–5 min).

An additional analyses in L5 ligated animals were performed to examine the degree of relationship between mechanical paw withdrawal threshold to weighted formalin pain score, paw elevation, or paw licking during the second phase (20–60 min) of the formalin test. There is no clear relationship between mechanical paw withdrawal threshold and any behavioral index of formalin responses. Indeed, analyses revealed a lack of a statistically significant relationship of mechanical paw withdrawal threshold to either the weighted formalin pain score ($P > 0.70$), paw elevation ($P > 0.15$), or paw licking ($P > 0.30$).

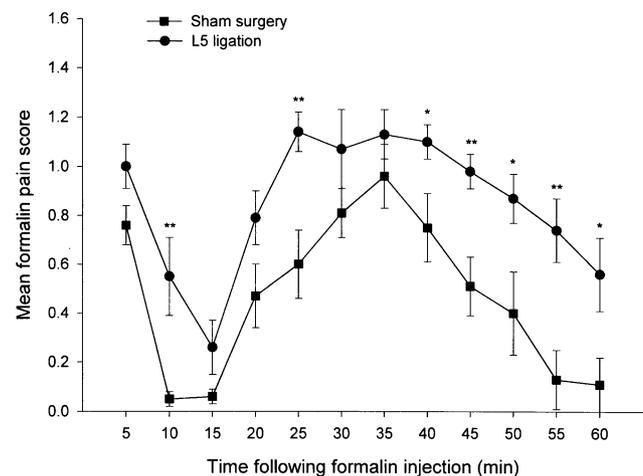


Fig. 2. Mean (\pm SEM) weighted formalin pain score across the 60-min formalin test period for animals that had nerve injury produced by ligation of the L5 spinal nerve ($n = 9$) or served as sham surgery animals ($n = 9$). Enhanced formalin pain responses were revealed at virtually every time period during the second phase of the formalin test (20–60 min). * $P < 0.05$; ** $P < 0.01$ vs. sham surgery.

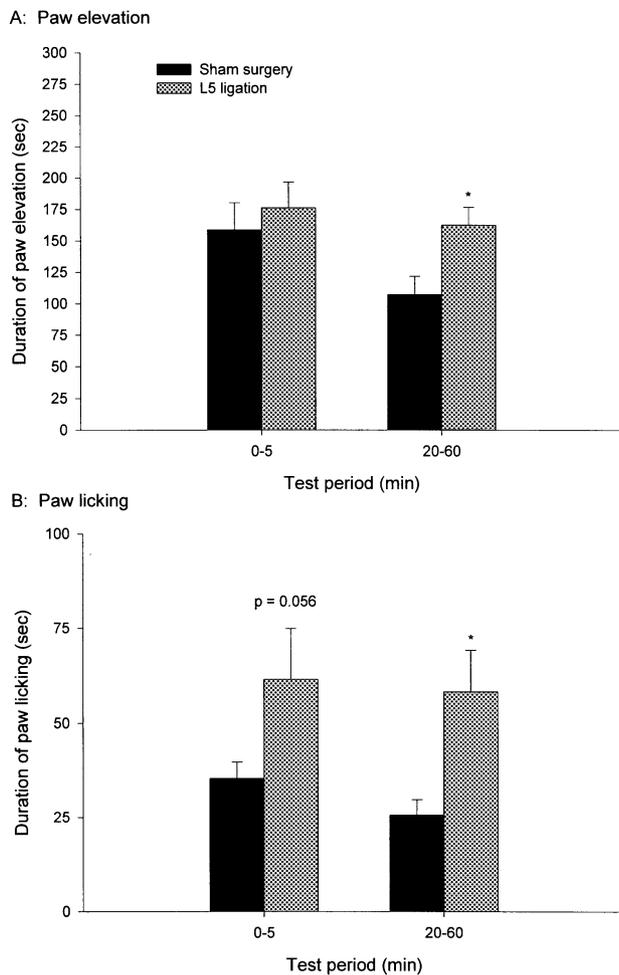


Fig. 3. Mean (\pm SEM) paw elevation (A) or paw licking (B) for the first phase of the formalin test (phase 1, 0–5 min) or the second phase of the formalin test (phase 2, 20–60 min) for animals that had nerve injury produced by ligation of the L5 spinal nerve ($n = 9$) or served as sham control animals ($n = 9$). Enhanced paw elevation and paw licking responses were revealed during the second phase of the formalin test. There is a strong trend towards enhanced paw licking response during the first 5 min of the formalin test. * $P < 0.05$ vs. sham surgery.

4. Discussion

The present results demonstrate that pain responses during the second phase of the formalin test were significantly increased following ligation of the L5 spinal nerve compared to sham surgery formalin injected animals. The enhanced responding was evident as an increase in paw elevation and paw licking in the inflamed, nerve injured paw. This finding, together with the relatively small and non-statistically significant enhancement of responding during the initial 5-min of the formalin test, indicates that hyperalgesia to an inflammatory condition develops during a neuropathic pain condition and supports a previous report of enhanced chemogenic pain following loose ligature of the sciatic nerve (Bennett and Xie, 1988) and diabetic peripheral neuropathy (Ceseña and Calcutt, 1999).

Ligation of the L5 spinal nerve is a well-characterized

animal model of neuropathic pain (Kim and Chung, 1992). The procedure results in ongoing and stimulus-evoked behaviors that are thought to reflect characteristics of clinical pain conditions, such as allodynia (reduced withdrawal thresholds for normally innocuous stimulation), hyperalgesia (enhanced withdrawal response to normally nocuous stimulation), and spontaneous pain (reluctance to put weight on the paw, etc.). Following tight ligation of the L5 spinal nerve, it was found that the average difference in withdrawal thresholds between the ligated and non-ligated paws was 249 mN (Fig. 1). This finding is a clear indication of allodynia and replicates previous findings that tight ligation of the L5 spinal nerve enhances behavioral responses to mechanical stimulation (Kim and Chung, 1992, LaBuda and Fuchs, 2000).

The mechanism(s) that are responsible for the enhanced responding to mechanical stimulation following ligation of the L5 spinal nerve are still largely unknown. One line of evidence suggests that neuropathic pain is the result of pathology at the site of nerve injury (Devor and Seltzer, 1994). In this model, ectopic impulses from the injured myelinated afferents (Michaelis et al., 1995) lead to a state of central sensitization, resulting in enhanced pain. The primary evidence supporting this proposal is that dorsal rhizotomy performed after nerve injury eliminated behavioral signs of neuropathic pain (Sheen and Chung, 1993; Yoon et al., 1996). A recent finding, however, suggests that mechanical hyperalgesia can persist in the absence of input from injured afferents (Li et al., 2000). According to the model proposed by Li et al. (2000), nerve ligation leads to Wallerian degeneration of peripheral L5 nerve fibers, and the subsequent alterations in the environment surrounding the intact nerve fibers from adjacent spinal nerves underlie the observed mechanical hyperalgesia.

Whether similar mechanisms account for the increase in formalin responses following ligation of the L5 spinal nerve remains unknown. However, the lack of a significant relationship between mechanical paw withdrawal threshold and formalin test measures (i.e., weighted pain score, paw elevation, paw licking) following L5 ligation seems to indicate that inflammatory and mechanical hyperalgesia are mediated by distinct mechanisms. Under normal conditions, phase 1 formalin pain responses are thought to reflect activity that is prominent in $A\beta$, $A\delta$ and high threshold C nociceptor afferent fibers. Phase 2 behavioral responses likely reflect activity in MIA fibers and activity of $A\delta$ and C fibers with receptive fields outside of the injection zone (Puig and Sorkin, 1995). Therefore, MIAs appear to be selectively active during the second period of the formalin test (Puig and Sorkin, 1995) and enhanced responses in these afferents following L5 ligation might account for the enhanced inflammatory response during the second phase of the formalin test. The mechanism by which MIAs become sensitized is unknown, but might be due to peripheral (i.e., enhanced adrenergic sensitivity in injured MIAs following nerve injury and/or altered environmental condi-

tions of uninjured MIAs caused by Wallerian degeneration following nerve injury) or central sensitization.

Regardless of the mechanisms responsible for enhanced formalin pain responses following nerve injury, the present results clearly demonstrate that enhanced inflammatory responses can develop during a neuropathic condition. As far as we know, this is the first report of inflammatory hyperalgesia in a rodent model of neuropathic pain induced by ligation of the L5 spinal nerve. Further exploration of the mechanisms responsible for this finding might be useful for understanding of mechanisms surrounding clinical pain conditions in which nerve injury and inflammatory conditions might both present.

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