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Experimental Neurology 191 (2005) 128–136

Experimental Neurology

www.elsevier.com/locate/yexnr

Ectopic discharges from injured nerve fibers are highly correlated with tactile allodynia only in early, but not late, stage in rats with spinal nerve ligation

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Received 26 April 2004; revised 21 August 2004; accepted 20 September 2004
Available online 19 November 2004

Abstract

It is widely accepted that ectopic discharges originated from injured sites and dorsal root ganglion (DRG) neurons after peripheral nerve injury contribute to neuropathic pain. However, it has been recently shown that ectopic discharges were not always necessary for neuropathic pain. In the present study, we aim to further examine the role of ectopic discharges in neuropathic pain in a spinal nerve ligation (SNL) model. With teased fiber recordings in vivo, the characteristics of ectopic discharges were observed over 14 days after SNL, and the correlation between ectopic discharges and tactile allodynia was analyzed. It was observed that ectopic discharges have three firing patterns (tonic, bursting, and irregular) after SNL, and proportions of these three patterns changed dynamically over time. The tonic and bursting types were dominant in the first 24 h following SNL, while the irregular type became the only pattern in the late stage (day 14). The average frequencies of ectopic discharges and the percentage of active filaments also changed over time, reaching the peak 24 h after SNL and then declined gradually. Ectopic discharges were highly correlated with tactile allodynia in the first 24 h following SNL, but surprisingly, not in the late stage of days 1 to 14. These findings suggest that ectopic discharges may be crucial in the triggering of neuropathic pain in the early stage, but their importance become more limited over time.

Keywords: Ectopic discharge; Dorsal root ganglion; Neuropathic pain; Spinal nerve ligation; Teased fiber recording; Tactile allodynia

Introduction

Neuropathic pain is characterized by spontaneous pain and tactile allodynia (pain evoked by normally innocuous mechanical stimuli). The mechanisms underlying neuropathic pain are largely unclear. After peripheral nerve injury, the ectopic discharges developed in the injured sites (Tal and Eliav, 1996; Wall and Devor, 1983; Wall and Gutnick, 1974a,b) and in the dorsal root ganglion (DRG) neurons (Liu et al., 1999; Liu et al., 2000a,b) are believed to play a crucial role in the generation and maintenance of neuropathic pain (Liu et al., 2000a). The ongoing ectopic discharges may initiate and maintain spinal central sensitization that amplifies inputs from the residual intact afferent nerves. Eliminating afferent ectopic firings has been shown to relieve spontaneous pain and tactile allodynia or thermal hyperalgesia in rat models (Sheen and Chung, 1993; Yoon et al., 1996). However, there are controversial reports. The ectopic discharges from the injured DRG are not necessary for the development and maintenance of neuropathic pain, at least in the spinal nerve ligation (SNL) model (Li et al., 2000). In contrast, spontaneous discharges from uninjured C-fiber played a key role in monkeys after SNL (Ali et al., 1999; Wu et al., 2001, 2002). Furthermore, accumulating evidences suggest that the spinal cord alone can lead to tactile allodynia in the absence of peripheral ectopic

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doi:10.1016/j.expneurol.2004.09.008

discharges (Tsuda et al., 2003). Taken together, the role of ectopic discharges originating from injured DRG neurons in neuropathic pain needs to be further explored (Gold, 2000).

In the present study, we aim to examine the role of ectopic discharges in tactile allodynia using the SNL model of neuropathic pain. Combined electrophysiological and behavioral methods are used to monitor the development of tactile allodynia and ectopic discharges after SNL over a relatively long period of time. The ectopic discharges are characterized and their correlation with behavioral allodynia is analyzed in the early as well as the late stages.

Materials and methods

Animals

Male Sprague–Dawley rats weighing 200–250 g were used. They were provided by the Department of Experimental Animal Sciences, Health Science Center Peking University, and habituated for 7 days before experiments. The animals had free access to food and water during experiments and were maintained on natural day/night cycles. All protocols were approved by the Animal Use and Care Committee of Peking University.

SNL model

Ligation of the left L5 spinal nerve was performed as described by Kim and Chung (1992). Briefly, the rats were anesthetized with 10% chlorohydrate (0.3 ml/100 g body weight) and placed in a prone position. An incision was made into the left of the spine at the L4–S2 level. The left L5 spinal nerve was carefully isolated and tightly ligated with 6–0 silk suture 5–10 mm distal to the DRG, and then cut approximately 2 mm distal to the suture. In sham-operated rats, the left L5 spinal nerve was without ligation.

Behavioral test with von Frey hair for mechanical allodynia

The mechanical sensitivity of the left hind paw was tested before and 1–14 days after nerve ligation. The experimenter was blind with respect to the condition of the rats (SNL vs. sham operation). The tests were performed once every 3 h in the first 24 h after operation. The 50% paw withdrawal threshold (PWT) in response to a series of von Frey filaments was determined by the up–down method (Chaplan et al., 1994). The rat was placed on a metal mesh floor covered with an inverted clear plastic cage (18 × 8 × 8 cm) and allowed a 15-min period for habituation. Eight von Frey filaments with approximately equal logarithmic incremental (0.224) bending forces were chosen (0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, and 15.10 g). Each trial started with a von Frey force of 2.00 g delivered perpendicularly to the plantar surface of the left hindpaw for about 2–3 s. An abrupt withdrawal of the foot during stimulation or immediately after the removal of the hair was recorded as a positive response. Whenever there was a positive or negative response, the next weaker or stronger filament was applied, respectively. This procedure was done until six stimuli after the first change in response had been observed. The 50% PWT was calculated using the following formula: 50% PWT = $10^{(X+kd)}/10^4$, where X is the value of the final von Frey filament used (in log units), k is a value measured from the pattern of positive/negative responses, and k = 0.224, which is the average interval (in log units) between the von Frey hairs (Dixon, 1980). If an animal responded to the lowest von Frey hair, a value of 0.25 g was assigned. If an animal did not respond to the highest von Frey hair, the value was recorded as 15.0 g.

Extracellular electrophysiological recording of ectopic discharges in vivo

Rats with ligation and cut of the L5 spinal nerve and rats with sham operation were anesthetized with urethane (1.5 g/kg, ip). A tracheotomy was performed, electrocardiogram and heart rate were monitored, and rectal temperature was kept constant at 37–38°C using a feedback-controlled radiant heater. No paralytic agents were used. L5 dorsal root was exposed in a lower lumbar laminectomy and covered with warmed paraffin oil (36°C) in a pool formed by skin flaps. The teased fiber recording method was used to record the ectopic afferent discharges entering the spinal cord along the dorsal root. Most of the dorsal muscles supplied by the dorsal ramus of the L5 spinal nerve were removed during the laminectomy. The L5 dorsal root was carefully examined and any communicating branches between L5 and neighboring dorsal roots were cut to eliminate any afferent firing from normal nerves. Nevertheless, residual dorsal ramus fibers, identified by their receptive fields on the lower back, were occasionally encountered in the dorsal root. They were not included in the count of fibers with ectopic activity.

Fine axon bundles (microfilaments) were teased from the dorsal root using specially honed No. 5 jewelers forceps (Fine Science Tools, Inc., Swiss). Microfilaments, cut centrally but in continuity with the DRG distally, were separated from the dorsal root near its point of entry into the spinal cord, 25–30 mm central to the DRG. The cut end of the microfilament was placed on a platinum recording electrode referenced to a nearby indifferent electrode. Each microfilament was observed passively for ≥2 min. If any spontaneous action potentials occurred within this period, observation was extended and the frequency and pattern of firing were registered. Microfilaments often contained zero to three spontaneously active axons. Spike discrimination was performed by a window discriminator and controlled by means of an electronic delay unit. The number of spontaneously active nerve fibers in each microfilament and their firing patterns

were measured by observing the different spike heights in the ectopic discharges. Data were captured and analyzed with the Micro1401 mk II and Spike 2 software (Cambridge Electronic Design, UK) as previously described (Xing et al., 2003).

In each experiment, the dorsal root was divided into fine microfilaments, each of them having a diameter of about 30–40 μm (judged by comparing with the diameter of the recording electrode, which was 100 μm) (Ma et al., 2003). At least 40 microfilaments were recorded in each rat. Two parameters of each unit were chosen as indicators. One is the proportion of filaments with at least one spontaneous unit; the other is the average frequency of ectopic discharges. Electrophysiological recording was carried out at 11 time points after SNL operation (hours 0–4, 5–8, 9–12, 13–16, 17–20, and 21–24; and days 2, 3, 5, 7, and 14) and at two time points after sham operation (hours 21–24 and day 14). Each group included 3–10 rats.

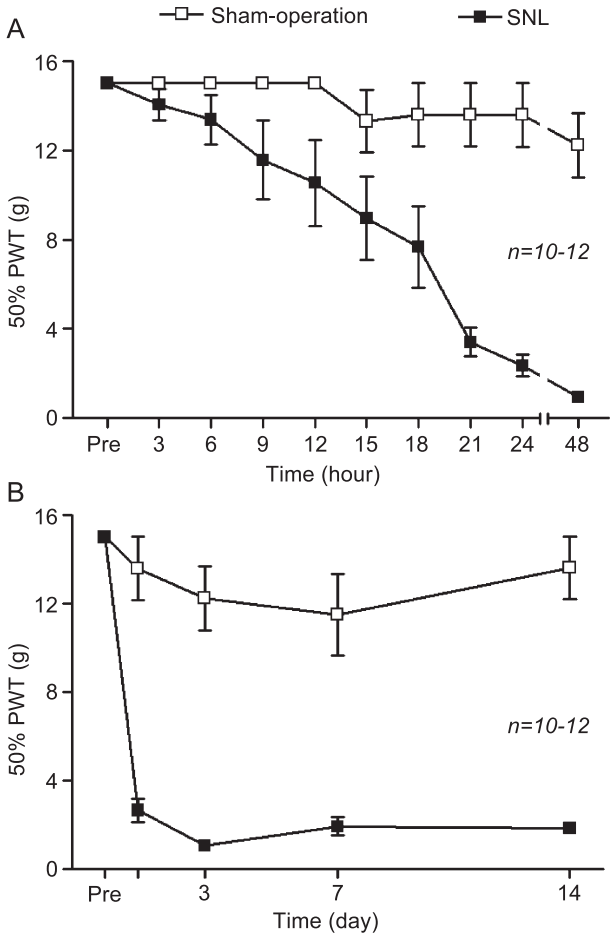

Fig. 1. Tactile allodynia developed after spinal nerve ligation (SNL). (A) Behavioral tests once every 3 h after SNL operation and sham operation. Note that 50% paw withdrawal threshold (PWT) significantly declined 15 h after SNL compared with pre-operation, while there was no significant changes in sham-operated rats. (B) Behavioral tests performed within 14 days. There was a reduced 50% PWT maintained at approximately the same level from days 1 to 14 after SNL ($P < 0.01$, compared with pre-operation). Data represented as mean ± SEM. Pre: Pre-operation control.

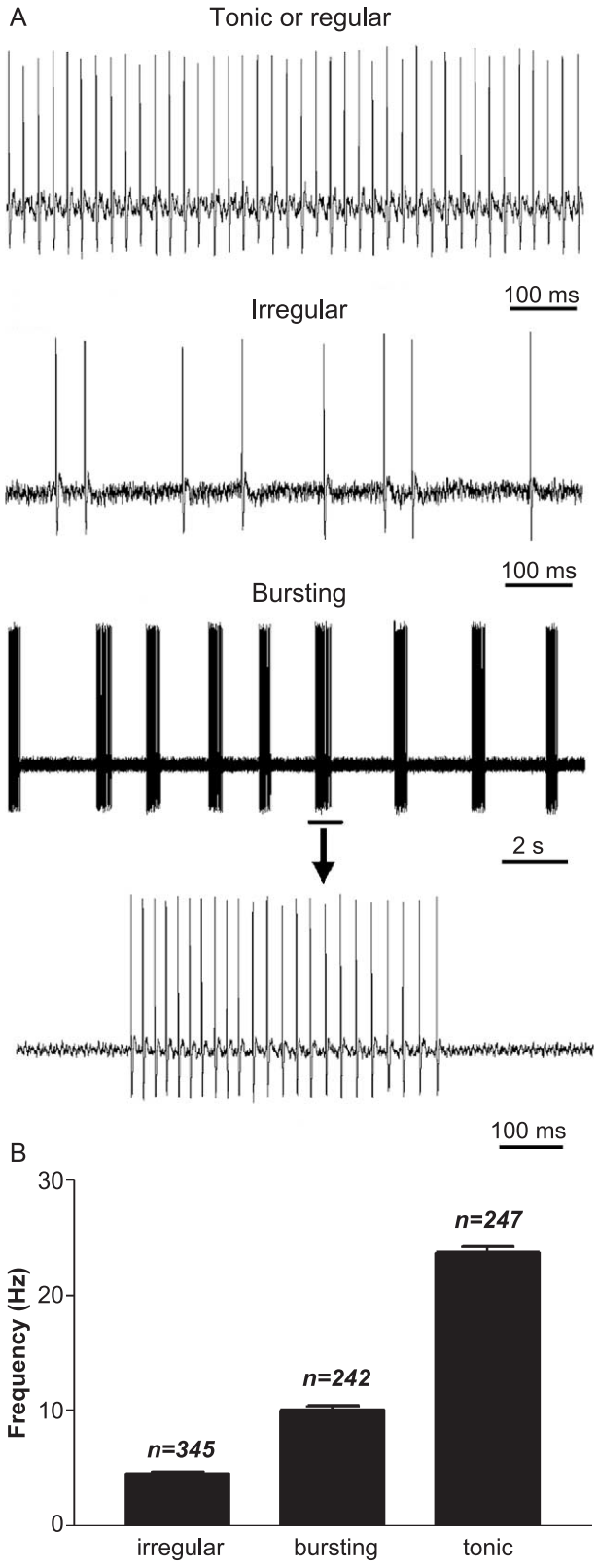

Fig. 2. Three different firing patterns with different frequencies after spinal nerve ligation. (A) Typical examples of ectopic discharges of three different firing types: tonic, bursting, and irregular. (B) Average frequencies of the three patterns: tonic > bursting > irregular.

Statistical analysis

Data are expressed as mean ± SEM. Repeated measures analysis of variance (ANOVA) followed by Dunnett's test and Student's *t* test were used for data analysis. *P* values less than 0.05 were considered to be significant.

Results

Mechanical allodynia by von Frey hairs

50% PWT was significantly reduced from 15.0 ± 0 g at pre-operation day to 8.96 ± 1.88 g at hour 15 ($P < 0.05$) (Fig. 1A), and continued to reduce to 0.97 ± 0.12 g on day 2 after SNL operation ($P < 0.001$), indicating the development of tactile allodynia. From days 1 to 14 (the end of observation), tactile allodynia was checked at days 1, 3, 7, and 14. The PWT was within the range of 1.0–2.5 g throughout the observation period (Fig. 1B). These data are similar to our previous observations (Wang et al., 2002) as well as those reported by others (Liu et al., 2000a). In contrast, there was no significant change of 50% PWT in sham-operated rats (Figs. 1A and B).

Frequencies and proportions of different firing patterns of ectopic discharges

After spinal nerve ligation, ectopic discharges could be recorded in many injured fibers. Almost all of the discharges recorded can be divided into three different patterns (Fig. 2) based on the interspike interval (ISI) histograms and their frequencies: (1) continuous ongoing "tonic" pattern with uniform ISI and frequencies ranging from 14.6 to 58.2 Hz with a median of 22.5 Hz, (2) interrupted bursting "on–off" type with frequencies ranging from 0.4 to 33.9 Hz, and (3) irregular discharges with low frequencies ranging from 0.03 to 15 Hz. The average frequencies of these firing patterns were 23.7 ± 0.5 Hz ($n = 247$), 10.0 ± 0.4 Hz ($n = 242$), and 4.5 ± 0.2 Hz ($n = 354$), respectively.

The proportion of the three firing patterns varied over time. As shown in Fig. 3, the proportion of tonic pattern reached peak at hour 16–24, then gradually decreased in the following 7 days, and disappeared at day 14. Bursting was dominant at hour 8–12, and its proportion gradually declined over 7 days and fell to zero at day 14. The proportion of irregular pattern gradually increased over the entire observation period, becoming dominant from day 5 and was the only pattern on day 14. Thus, bursting and tonic were the dominant patterns in the first 24 h, but then declined as time progressed, while irregular gradually increased at the same time.

Dynamic changes of ectopic discharges over time

In the first 4 h after ligation and cut of spinal nerves, no ectopic discharge was recorded at all (Fig. 4). At hours 4–8 after SNL operation, both the proportion of active filaments (6.2 ± 3.4%, $n = 10$) and the frequencies (7.8 ± 1.3 Hz, $n = 27$) of ectopic discharges were very low. In fact, ectopic activities could be recorded only in 4 out of 10 rats. The proportion then increased rapidly over time, reaching to the highest (79.0 ± 2.0%, $n = 3$) ($P < 0.001$) at hours 20–24. At the same time, the average frequencies of ectopic discharges

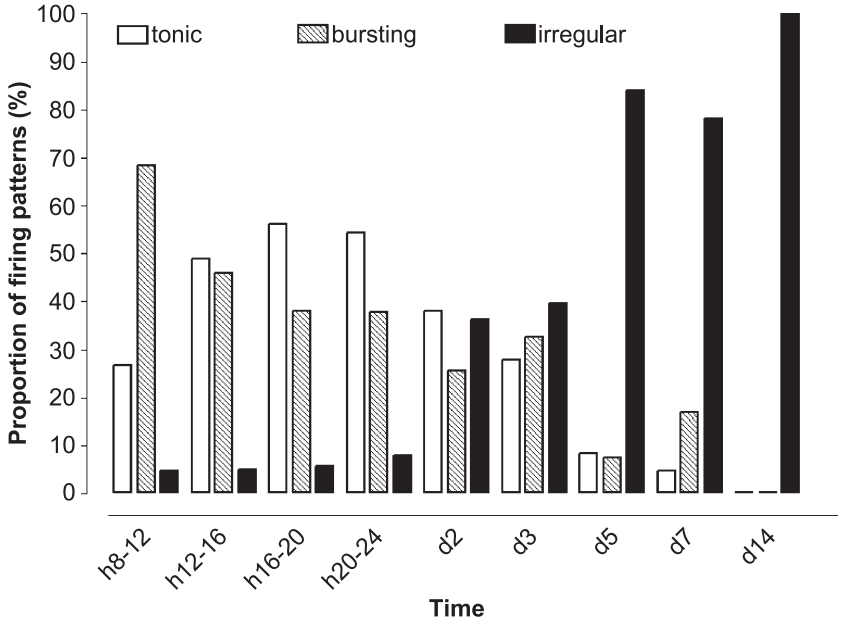

Fig. 3. Changes of patterns of ectopic discharges at different time points after spinal nerve ligation (SNL). h and d represent hours and days after SNL, *n* for 8–12 h, 12–16 h, 16–20 h, 20–24 h, 2 d, 3 d, 5 d, 7 d, and 14 d is 41, 88, 105, 142, 174, 153, 95, 143, and 139, respectively. Illustrations refer to text in the Results section.

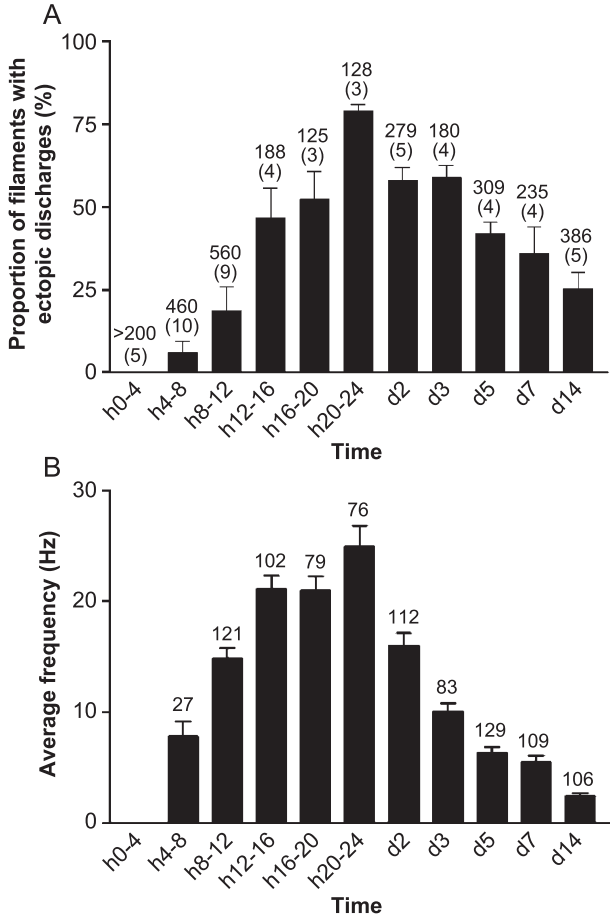

Fig. 4. Dynamic changes of ectopic discharges after spinal nerve ligation (SNL). (A) Proportions of filaments with ectopic discharges. The proportion reached peak at hours 20–24 post-operation then gradually declined. (B) Average frequencies of ectopic discharges. Similar tendency was found as in (A). Data represented as the mean ± SEM. Numbers above the columns refer to the microfilaments sampled, and the numbers in the parentheses represent rats used.

also increased significantly to 24.9 ± 1.9 Hz (n = 76) (P < 0.001).

On the other hand, as shown in Fig. 4, both proportions and frequencies of ectopic discharges decreased quickly after day 1. Proportions of active filaments and average frequencies are 25.5 ± 4.9% (n = 5) and 2.5 ± 0.2 Hz (n = 106), respectively, at day 14, both of them are significantly lower than those at hours 20–24 (P < 0.001), indicating that the excitability of the injured DRG neurons diminished profoundly during this period.

Correlation between ectopic discharges and tactile allodynia

As shown in Fig. 4, ectopic discharges at hours 20–24 after SNL operation reached peak. The proportion of active filaments and the average frequencies of ectopic discharges were chosen to analyze the relationship between ectopic discharges and tactile allodynia. As shown in Fig. 5, changes of ectopic activities in the first 24 h are highly correlated with 50% PWT. The higher the proportions of active filaments and average frequencies, the lower the 50% PWT. However, in contrast to the high correlation within the first 24 h, from days 1 to 14, there is no relationship between ectopic discharges and tactile allodynia (Fig. 6).

Spontaneous activities of afferent fibers in rats with sham operation

In rats with sham operations, the average frequency at hours 21–24 (27.0 ± 1.7 Hz, n = 83) is similar to that at day 14 (26.3 ± 2.1 Hz, n = 75). No significant difference was observed (P > 0.05). Likewise, the proportion of active filaments at hours 21–24 (66.1 ± 6.5%, n = 3) is not significantly different from that at day 14 (78.1 ± 10.4%, n = 3) (P > 0.05). In sham-operated rats, only two patterns of firing were recorded, the tonic (174/225, 77.3%) and irregular (51/225, 22.7%) types.

Discussion

Spinal nerve ligation is a useful model for neuropathic pain, and there are a relatively few papers reporting ectopic discharges in this model (Liu et al., 2000a). The present study examined changes of ectopic discharges and analyzed their relationship to mechanical allodynia over 14 days after SNL. The main findings are as follows: (1) ectopic discharges of three different firing patterns developed following SNL. The proportions and frequencies changed dynamically over time; (2) the onset of the tactile allodynia within 24 h after SNL is highly correlated with ectopic discharges, but from days 1 to 14, no correlation was found between tactile allodynia and ectopic discharges. Both the proportion of active filaments and the average frequencies significantly declined as time progressed, although tactile allodynia was maintained at a stable level.

Characteristics of ectopic discharges after SNL

Almost all of the ectopic discharges can be divided into three firing patterns, and these results are supported by other previous studies in vivo or in vitro (Liu et al., 1999; Liu et al., 2000a,b). Frequencies of ectopic discharges and proportions of active filaments reached peak approximately at hours 20–24 after SNL, suggesting that the excitability of DRG neurons is highest at this time. Thus, the patterns of ectopic discharges in the spinal nerve ligation model differ, to a large extent, from those in the sciatic nerve transection model, since in the latter case, ectopic discharges from injured DRG neurons developed late (Wall and Devor, 1983). In addition, almost all ectopic discharges from DRG neurons belong to irregular pattern after sciatic nerve axotomy (Liu et al., 2000a; Wall and Devor, 1983). From this point of view, we should be careful when comparing

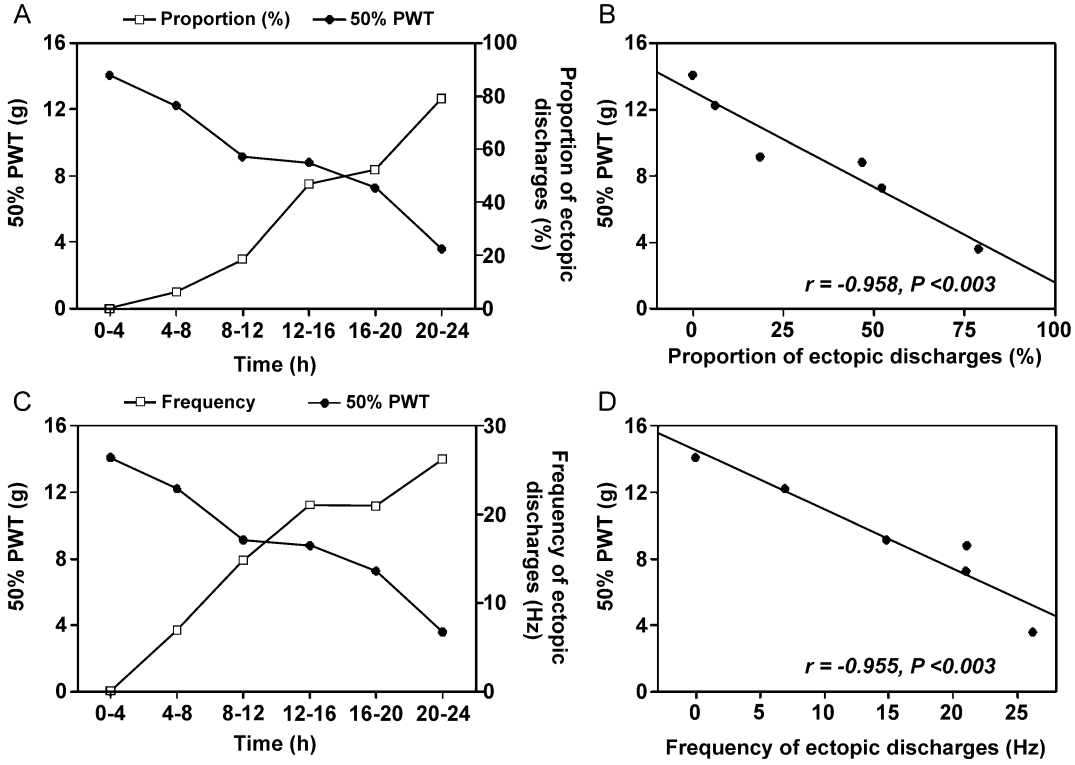

Fig. 5. Correlation analysis between tactile allodynia and ectopic activities within the first 24 h following spinal nerve ligation (SNL). 50% paw withdrawal threshold (PWT) declined when proportions of filaments (A) or the average frequencies of ectopic discharges (C) increased. High negative correlation is found between tactile allodynia and proportion of active filaments (B) ($r = -0.958$, $P < 0.003$), or between tactile allodynia and average frequencies of ectopic discharges (D) ($r = -0.955$, $P < 0.003$).

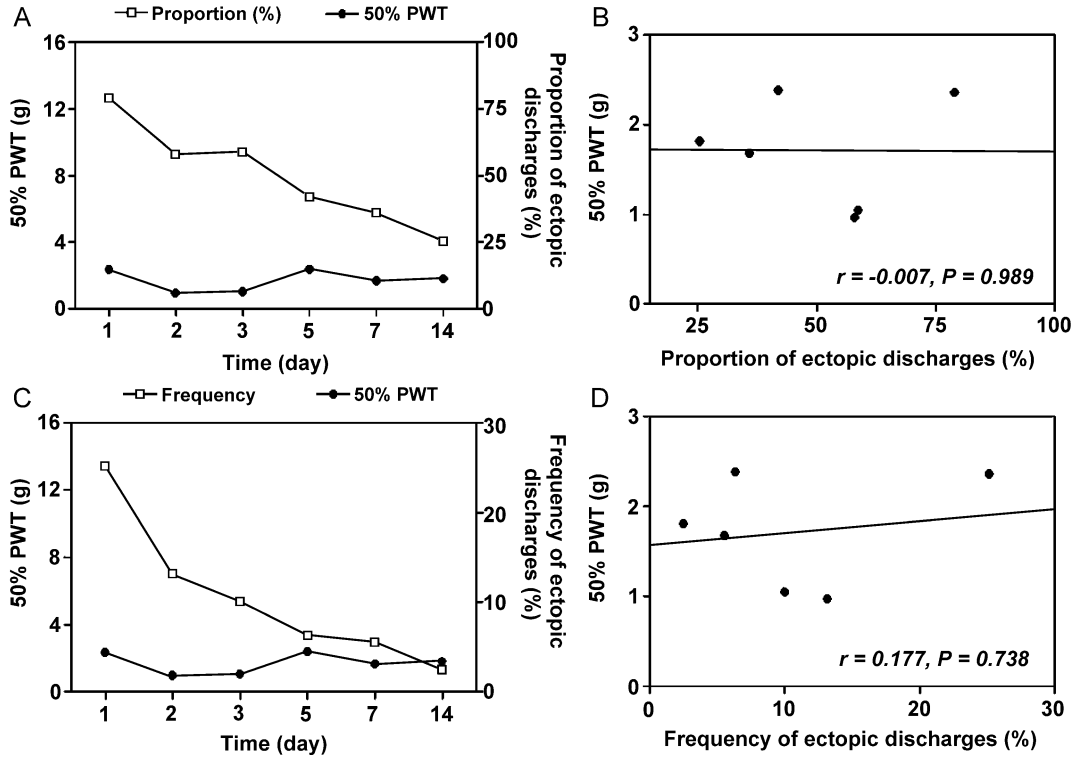

Fig. 6. Correlation analysis between tactile allodynia and ectopic activities from days 1 to 14 following spinal nerve ligation (SNL). 50% PWT changed very little from days 1 to 14 after SNL (A and C) while proportions of filaments with ectopic discharges (A) or the average frequency of ectopic discharges (C) declined significantly. No correlation is found between tactile allodynia and proportion of active filaments (B) ($r = -0.007$, $P = 0.989$) or of average frequencies of ectopic discharges (D) ($r = 0.177$, $P = 0.738$).

experimental results between different types of nerve injury models (Liu et al., 2000a).

In sham-operated rats, we also recorded spontaneous discharges arising likely from proprioceptive afferents. Both the average frequencies and proportions of active filaments in sham-operated rats are similar to those at day 1 in the SNL group. However, the average frequencies of ectopic discharges and the proportions of active filaments in the SNL group changed dynamically over time, whereas in the sham operation group, both parameters at day 1 are similar to those at day 14. These data indicate that the change of ectopic discharges in the SNL group results from the ligation of spinal nerves, but not from other factors, for example, inflammation. In addition, only two firing patterns (tonic and irregular) were recorded in sham-operated animals in contrast to the three patterns in the SNL rats. In 225 active units, we found that 174 units belong to tonic type and 51 belong to irregular type. Finally, unlike the SNL group, the proportion of firing patterns in the sham group also did not change over time.

It is to be noted that in addition to the firing frequencies and proportions of active filaments with ectopic discharges, the types of spontaneously active fibers are also very important, because the different types of fibers might have different physiological and pathophysiological significance in the development of neuropathic pain. As reported previously (Boucher et al., 2000; Han et al., 2000), almost all ectopic discharges in SNL rats originate from A fibers, but not the C fibers.

Are the ectopic discharges emanating from the injured peripheral nerves necessary for tactile allodynia after SNL?

We observed robust discharges at hours 20–24 after SNL. This is in agreement with others (Liu et al., 2000a). The high correlation to the behavioral changes at this early stage suggests that ectopic discharges may play an essential role in the initiation of neuropathic pain (Fig. 5). Although the correlation did not prove a causal relationship, this assertion is also supported by the finding that L5 dorsal rhizotomy almost completely eliminated ongoing pain and allodynia after SNL (Sheen and Chung, 1993; Yoon et al., 1996). Moreover, the development of tactile allodynia in the SNL model was not affected by C-fiber desensitization with resiniferatoxin (RTX) (Ossipov et al., 1999), suggesting a role for the residual myelinated fibers, and finally, several other studies have also established a relationship between tactile allodynia and ectopic discharges (Gold, 2000; Han et al., 2000; Liu et al., 2000a).

The most interesting and important finding in the present study is that ectopic discharges were highly correlated to tactile allodynia only within the first 24 h after SNL, but not in the late stage up to 14 days post-operation. Thus, in the days 1–14 after SNL, there was no correlation at all between ectopic discharges and tactile allodynia, which indicate that ectopic discharges may not be necessary in the late stage (i.e., maintenance) of neuropathic pain.

In addition to ectopic discharges, some other factors have been implicated in the development and maintenance of neuropathic pain. Our recent results (Xing et al., 2003) found that, at days 7–14 in SNL rats, the thresholds of C-fiber-evoked field potentials in the dorsal horn were significantly lower and the amplitudes tended to be higher. The threshold for C-fiber to initiate spinal long-term potentiation (LTP) was also decreased significantly. This finding suggested that the spinal dorsal horn was sensitized after SNL. Other reports also supported a role of the spinal LTP in neuropathic pain (Ikeda et al., 2003; Randic et al., 1993; Sandkuhler and Liu, 1998; Svendsen et al., 1997; Xing et al., 2003). Additionally, several other reports showed convincing evidences that spontaneous activities from uninjured C-fiber nociceptors or axons participated in neuropathic pain (Ali et al., 1999; Wu et al., 2001, 2002). This spontaneous activity was proposed to be due to Wallerian degeneration of L5 axons (Ramer et al., 1997; Wu et al., 2001, 2002). Furthermore, there is a redistribution of sodium channel $Na_v1.8$ in the uninjured axons and knockdown of $Na_v1.8$ gene reversed neuropathic pain in the SNL model (Gold et al., 2003). Therefore, spontaneous activities from uninjured C-fiber nociceptors or axons may be another source of ectopic discharges to sensitize the spinal dorsal horn in late stage. Descending facilitation system has also been proposed to play an essential role in the maintenance of neuropathic pain of SNL rats (Burgess et al., 2002). Injection of lidocaine to the rostral ventromedial medulla (RVM) blocked SNL-induced tactile and thermal hypersensitivity at days 6–12, but not before day 3 (Porreca et al., 2002), indicating that the descending facilitation is a key element in the maintenance, but not the initiation, of neuropathic pain (Burgess et al., 2002; Millan, 2002). Other central factors, including wind-up (Vikman et al., 2001; Woolf, 1996), activated glial cells (Jin et al., 2003; Tsuda et al., 2003; Watkins and Maier, 2002; Watkins et al., 2001), as well as reorganization of nerve terminals in spinal dorsal horn, may also participate in the maintenance of neuropathic pain in the late stage.

High correlation between ectopic discharges and neuropathic pain behavior was also reported in a relatively long time period. For example, Han et al. (2000) reported that ectopic discharges and neuropathic pain behaviors declined as post-operative time progressed over a time period of 26 weeks. Such discrepancy with our results suggests that the relationship between ectopic discharges and pain behavior is complex. One important source of such difference is strain of rats. Male Sprague–Dawley rats originally from Charles River Laboratories are used in the present experiment, while Wistar rats (Liu et al., 2000a,b) or Sprague–Dawley rats from different vendors were used in other studies (Han et al., 2000). We and others have reported that strain difference influences ectopic discharges and pain behaviors (Lee et al., 1997; Lovell et al., 2000; Wan et al., 2001; Huang et al.,

2002). In fact, neuropathic pain behaviors in our experiments did not last as long as others (unpublished data).

Even though there was no correlation between the irregular discharges and the tactile allodynia after 24 h, we cannot conclude that these irregular ectopic discharges with low frequencies are definitely not important in the late stage. Rather, the deceased ectopic discharges may still play roles in the maintenance of neuropathic pain, or the relatively sparse, irregular ectopic discharges may still be sufficient to activate the sensitized spinal dorsal horn. Thus, ectopic discharges may still be indispensable to neuropathic pain in the late stage. Otherwise, it is difficult to explain why inhibition of ectopic discharges can suppress tactile allodynia (Lyu et al., 2000). It should be noticed, however, that in experiments mentioned above, only partial suppression of tactile allodynia was achieved when ectopic discharges were inhibited by TTX (Lyu et al., 2000), suggesting that ectopic discharges themselves cannot fully account for neuropathic pain behavior (Boucher et al., 2000). Furthermore, we also observed that some neuropathic pain rats of the SNL model displayed obvious tactile allodynia in the late stage despite lack of ectopic discharges (unpublished data). This clearly suggests that ectopic discharges are not essential for the maintenance of neuropathic pain beyond a certain time point after nerve injury.

In summary, the present study and many other recent works demonstrate that ectopic discharges are very important in the initiation of neuropathic pain in early stage. On the other hand, in the late stage, ectopic discharges from the injured fibers decreased significantly, indicating that they may play a limited role in the maintenance of neuropathic pain. We propose that central factors, including spinal dorsal horn sensitization and supraspinal descending systems, as well as neighboring uninjured unmyelinated nociceptors or axons play more important roles in the maintenance of neuropathic pain in the late stage after SNL.

Acknowledgments

This project was supported by grants to You Wan from the National Natural Science Foundation of China (30330026, 30170319, 30240059, 30470559) and the National Basic Research Program of China (G19990540002). The authors thank Dr. Xiao-Jun Xu of Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden, for his kind help in preparing the manuscript.

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