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CO_2 Laser Pulse-Evoked Nocifensive Behavior Mediated by C-Fibers

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1. Introduction

Most tests that assess pain in animals involve motor responses to noxious stimuli. This practice depends on the implicit premise of a strong relationship between nociception and motor activity [Le Bars et al., 2001, Smith, 1995]. Therefore, a behavior can and should be analyzed as thoroughly as any brain structure and function involving motor activity in neurological investigations. The importance of analyzing behavior has been demonstrated by several studies on the mechanisms of nociception. For example, Rousseaux et al. [Rousseaux et al., 1999] showed that patients with spinothalamic tract injury had a higher temperature threshold than normal in response to nociceptive stimuli and have pain but not heat sensations. Thermal and electrical stimulation of the affected side in these patients elicited a withdrawal reaction. Another example, demonstrated by Shyu et al. [2003], was conditioned CO_2 laser-evoked behavior in rats to distinguish emotional components from sensory conductive pathways [Kung et al., 2003].

The ideal model of pain stimulation should be nociceptive-specific, controllable, safe, and reproducible. Pain pathways are a part of the somatosensory system. Therefore, neurophysiological investigation of pain pathways is related to the recording of somatosensory-evoked responses. To test the integrity of nociceptive pathways with evoked nociceptive responses, the timing of the activation of nociceptive afferents must be precise for stimulus-locked averaging. Many experimental pain models that fulfill this requirement have been successfully used in pharmacological studies. Nevertheless, most of these models have limited clinical application for precise diagnosis. For example, pain has been elicited by cutaneous intense electrical stimulation, which activates peripheral fibers that respond to both noxious and innocuous stimulation. The drawback of electrical stimulation may be partially overcome by using various blocking techniques. Another approach is to induce nociception by activating tooth pulp, which is innervated mostly by Aδ- and C-fibers [Roos et al, 1982, Van Hassel, 1972]. Stowell [1974] used an electromechanically driven pin to produce cutaneous pain, and Mitchell and Hellon [1977] used gradual warming to induce cutaneous thermal stimulation. However, both methods can excite thermo- or mechanoreceptors in addition to nociceptors [Burgess & Perl, 1973].

Non-contact thermostimulation is required for the investigation of the neural mechanisms underlying pain sensation without concurrent activation of mechanoreceptors. To record thermal-evoked potentials, thermostimulation must be very brief to permit the time-locking of potential averaging to the sensory event. Many repetitions of the thermal stimulation

without habituation are necessary to improve the signal-to-noise ratio, and only nociceptors should be activated. Applying gas lasers, such as CO_2 and argon, to the skin satisfies most of the aforementioned criteria. A laser beam does not actually contact the skin; therefore, heat and thermosensitive nociceptors are selectively activated, while mechanoreceptors are unaffected [Kakigi et al., 1989]. Thus, for the experimental study of pain and clinical neurology diagnosis, only thermal stimulation of non-glabrous skin by brief laser pulses has gained widespread clinical use.

The use of short CO_2 laser pulses in the study of pain was introduced by Mor and Carmon [1975]. These pulses have several potential advantages as noxious stimuli. First, the laser is the source of very short and concentrated thermal energy that activates thermosensitive nociceptors. Second, in contrast to conventional radiant heat, short laser pulses induce a synchronous volley of impulses in afferent nerves, allowing repeated and simultaneous measurement of time-locked evoked neuronal responses at different levels of the nervous system. Their high power output produces steep heating ramps, which improve synchronization of afferent volleys and therefore allow the recording of time-locked events, such as laser-evoked potentials (LEPs) in the brain. The amplitude of LEPs on the human scalp is related to subjective reports of pain sensation [Bjering & Arendt-Nielsen, 1988, Bromm & Treede, 1984, Carmon et al., 1978]. These findings suggest that LEPs may be used as objective correlates of pain perception.

Laser-evoked cortical responses can be classified into two groups [Danneman et al., 1994, Isseroff et al., 1982, Kalliomaki et al., 1993]. The first group, mediated by Aδ-fibers, is more sensitive to pentobarbital-induced anesthesia [Shaw et al., 2001] and laser pulse energy level [Kalliomaki et al., 1993]. The second group, mediated by C-fibers, is widespread across the cortical surface and can be diminished by topical morphine application onto the lumbar spinal cord and reversed by naloxone [Kalliomaki et al., 1998]. Although the peripheral and central conductance mechanisms responsible for cortical LEPs have been investigated, the results have been inconsistent. For example, Devor et al. [1982] claimed that only C-fibers, and no Aδ-fibers, were activated by CO_2 laser pulses, according to single-unit recordings in peripheral nerves in rats. However, the Isseroff group [1982] studied cortical potentials in anesthetized rats and found that laser-evoked cortical potentials have a latency of approximately 40 to 55 ms, which is within the range of Aδ-fibers.

We previously administered short-pulse CO_2 laser stimulation to freely moving rats to measure nociceptive sensitivity [Shyu et al., 1995] and studied nocifensive behavior induced by a similar model [Fan et al., 1995]. Nocifensive behavior was subdivided into eight discrete response components, including flinching, foot elevating, licking, body movements, and foot movements. Following stimulation with higher laser energy, rats exhibited both an increase in the number of response types and frequency of specific responses. The results suggested that hierarchically organized responses in the nocifensive motor system are recruited to varying degrees by noxious stimuli with different intensities [Fan et al., 1995]. Additionally, the threshold for activation of the nocifensive behavior component “body movement” was lower than that of the behavior components “flinching” and “licking” [Fan et al., 1995]. Le Bar et al. [2001] proposed that stimulus intensity can determine which type of nerve fiber initiates the reaction. Treede et al. [1995] illustrated this concept by showing that the threshold of C-fibers was significantly higher, and conduction was slower, than that of Aδ-fibers. The differential activation of Aδ- and C-fibers and the difference in their physiology suggest that they play differential roles in nociceptive responses under specific stimulation conditions [Lawson 2002]. In humans, considerable evidence indicates that

distinct secondary pain sensation is driven by C-fibers [Ploner et al., 2002, Torebjork & Ochoa, 1990] and is activated by C nociceptors distributed in the rostral hypothalamus [Lumb, 2002]. The supraspinal circuits activated by C-fiber inputs can be linked to nocifensive behavioral patterns related to the relative contribution of these types of nociceptive afferents [Le Bars et al., 1976]. Therefore, nocifensive behaviors can be differentially elicited and mediated by different classes of nociceptors.

The aim of the present study was to test the hypothesis that nocifensive behavioral elements elicited by brief laser pulse stimuli are mediated by C nociceptors. We applied the neurotoxin capsaicin, which acts specifically on primary sensory afferent C-fibers, to selectively block activity. Capsaicin has been locally applied to the sciatic nerve of the hind legs in rats to examine whether cortical LEPs are conducted by C-fibers [Sun et al., 2006]. In the present study, we used a previously established nocifensive behavioral model in rats to study the correlation between cortical LEPs and the response components evoked by a short-pulse CO_2 laser beam and examined whether those response elements are nocifensive responses mediated by C-fibers.

2. Materials and methods

2.1 Subjects

A total of 68 male Sprague Dawley rats, weighing 250-350 g, were used for these experiments. Food and water were available *ad libitum*. Rats were housed singly in standard wire mesh cages in a temperature- and humidity-controlled environment (21-23°C, humidity 50%) with a 12 h/12 h light/dark cycle (lights on at 0600 h). All experiments were conducted during the light period in a quiet room. Animal care adhered to the guidelines of the Academia Sinica Institutional Animal Care and Utilization Committee and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23).

2.2 Implantation of chronic cortical and electromyogram electrodes

Rats were surgically prepared before being subjected to neurophysiological and behavioral experiments. The rats underwent surgery while under pentobarbital anesthesia (50 mg/kg, i.p.; Abbott Laboratories, Chicago, IL, USA). For recording of cortical-evoked potentials, electrodes were chronically implanted into the rats' brains. Three holes (1.0 mm diameter) were drilled in the skull, one on each side (coordinates: 3 mm lateral to bregma, approximately in the region of the primary somatosensory cortex, S1) and the third 2 mm behind lambda as a reference lead. Stainless-steel screws were tightly inserted into the holes so that their tips made contact with the dura mater overlying the cortical surface. A thin Teflon-coated stainless steel wire was soldered on the edge of the screw head and connected with one of eight legs of an integrated circuit (IC) chip receptacle. The screws were used as electrodes and anchoring points for the IC chip receptacle, which was affixed to the skull with dental cement.

For electromyography (EMG), two multi-strand stainless steel microwires were inserted into the hamstring muscle of the left hindlimb. Dental cement was applied to fasten the connection receptacle to the surface of the skull. The measurements were collected 1 week after surgery. The body weights of the treated rats were monitored between surgery and the recording day, which occurred 1 week after surgery. Post-surgical body weight gain served as an indicator of recovery from surgical stress. Rats that did not gain weight 1 week following surgery were excluded from the study.

2.3 Laser stimulation

The glabrous skin of the foot pad was stimulated with a high-intensity CO_2 laser beam (Carbon Dioxide Surgical Laser System, model 20 CH, Direct Energy, Inc., CA, U.S.A.). The system generated a laser radiation beam in the infrared spectrum (10.6 μm wavelength) and had adjustable power capable of producing peak laser power greater than 20 watts (W). The duration of the laser pulse was controlled by the duration of an externally triggered TTL pulse from a pulse generator (A-M systems Inc., USA). The laser beam was focused approximately 3 cm in front of the laser head, and the size of the beam was measured by burning a spot on heat-sensitive coated paper that was set at a distance indicated by a rod. In the acute spinal cord recording experiment, the laser tube was held by an experimenter or a rigid stainless steel stand.

2.4 Tactile stimulation

A 1.5 mm diameter stainless steel rod was attached to the voice coil of an 8 Ω, 15 W loud speaker as a tactile stimulus. The tactile stimulus was triggered and controlled by a TTL pulse from a pulse generator (A-M Systems Inc., USA). The movement of the coil transmitted to the rod produced an outward excursion of 5 mm.

2.5 Recording devices

A cable from above the cage was connected to the receptacle on the head of the animal. Electrical activity was recorded either from the cortex of freely moving animals or from the spinal cord of anesthetized animals and was amplified by differential AC amplifiers (0.1 Hz to 5 KHz, Model DAM70, WPI, USA). Analog signals were either displayed on a digital storage oscilloscope or sent to a IBM-PC-based data acquisition system for online A/D conversion (Metrabyte DAS-16F AD/DA interface card, USA) and digital analysis using Quick Basic Language. The signals were also stored on a hard disk for offline data analysis using a PC-based data analysis system.

2.6 Application of capsaicin to the sciatic nerve

After obtaining the evoked cortical potentials and behavioral responses, the left legs of the rats were treated with either capsaicin or vehicle solution. The rats (n = 26) were first anesthetized by halothane inhalation (2-3%). The sciatic nerves of the left legs were exposed and separated from the muscles and vessels by placing paraffin paper under them. A piece of cotton soaked with either vehicle (3% Tween-80 dissolved in 100% alcohol) or 1% capsaicin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in vehicle was applied directly to the nerve trunk for 45 min. After treatment, the cotton was removed, and the open muscle and skin were sutured. Rats were then given antibiotics intramuscularly and allowed to recover. Their cortical potentials and behavioral responses were tested 3 days after surgery.

2.7 Behavioral assessment and procedures

Control experiment. Stimuli had a fixed pulse duration of 30 ms and five graded intensities: 3, 5, 10, 15, and 20 W. Animals were randomly divided into five groups (n = 6/group), and each group received one of five stimuli. In each stimulation session, 20 laser stimuli of the same intensity were delivered. Stimulation protocols were chosen to establish a correlation between evoked cortical potentials and behavioral reaction patterns.

Capsaicin experiment. Twelve rats were randomly divided into two groups. One group received capsaicin treatment, and the other group received vehicle. The average laser- and tactile-evoked potentials were recorded before and after capsaicin or vehicle treatment. Both the treated and untreated hindpaws were stimulated with laser pulses (15 W intensity, 30 ms duration). The untreated side served as a control. The stimulus power was chosen based on our previous results [Shyu et al., 2003].

Testing procedures. Tests were performed on the day before capsaicin treatment (Day 0) and on Days 3, 5, and 14 after treatment. Animals were brought to the laboratory and placed into the test cage for 20 min before each test. This procedure familiarized the animal to the test environment to reduce exploratory activity during the test. The test cage measured 26.5 cm length × 21 cm width × 17 cm height and was constructed with transparent acrylic sheets with a 0.5 cm^2 stainless steel grid at the bottom. The cage was hung such that the bottom could be viewed by the experimenter to apply laser stimuli to the hindpaw. During a test session, the laser beam was projected from below the test cage to the plantar surface of the rat's hind footpad. The animals were unrestrained and free to react during the experiments. The experimental setup for laser stimulation, electrophysiological recording, and behavioral measurement in freely moving rats is shown in Fig. 1.

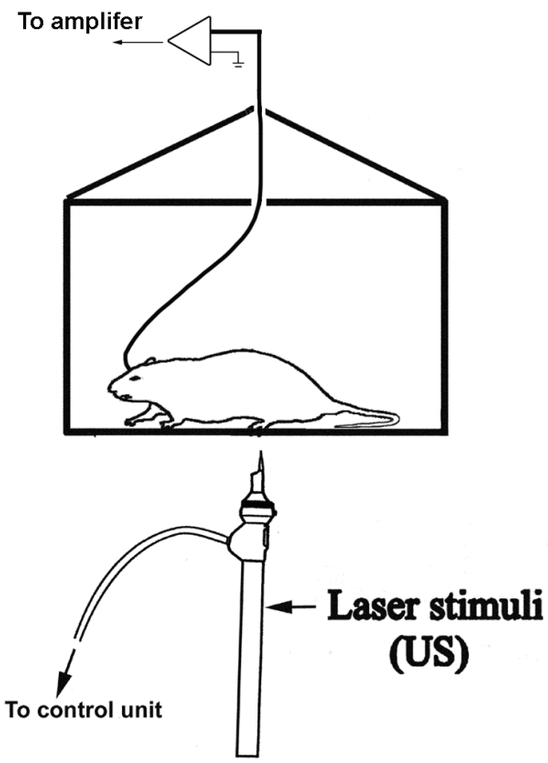

Fig. 1. Experimental setup for brief CO_2 laser pulse stimulation, electrophysiological recording, and behavioral assessment in unrestrained, freely moving rats.

Behavior analysis. Behavioral assessment was described in detail in our previous paper [Fan et al., 1995]. Briefly, behavior was observed for 2 min following each stimulus presentation because previous experience indicated that the animal returned to its resting status within 2 min. During this 2 min, whenever a response occurred, the frequency was counted as "1,"

no matter how many times it was repeated. The raw data following each stimulus consisted of whether a response occurred (1) or did not occur (0).
Five behavioral components were recorded and quantified: body movement, head turning, withdrawing, flinching, and licking. *Body movement* was defined as slow body motion, body turning, running, or exploration involving translocation of the body. *Head turning* included turning, shaking, or elevating the head. *Flinching* was recorded whenever a rat made a small abrupt jerking movement (< 1 cm) with its body. A *withdrawal* response was scored when an animal receded its body position by ≥ 1 cm away from the stimulus. *Licking* involved the rat retracting the paw by ≥ 1 cm and licking. Other complicated movements, such as tapping, that may have occurred together with a withdrawing or licking motion were recorded with the major movement that the complicated movement accompanied.
Twenty laser stimuli were applied during each session, and the maximum number of an observed behavioral component was 20 for each rat, even if the rat repeated the same behavior. The laser intensity used in the behavioral assessment was 10 W and 30 ms, based on our previous study [Fan et al., 1995].

2.8 Acute spinal cord preparation for electrophysiological recordings

Six rats that were not used for behavioral analysis were anesthetized by intraperitoneal injection of a mixture of ketamine (30 mg/kg) and rumpon (15 mg/kg). A PE-240 tube was inserted into the trachea for artificial respiration, and the left jugular vein was catheterized with a PE-50 tube for drug or fluid infusion. Body temperature was maintained at 37 ± 5°C by a homeothermo blanket system. During the recording experiments, animals were paralyzed and received artificial respiration. The end tidal CO_2 concentration was monitored and maintained in a range of 3-4%. Because the surgery and experiments may be prolonged, supplementary doses of ketamine (10 mg/kg) were administered intravenously to maintain the depth of anesthesia, which was monitored by pinching the tail of the rat. If a withdrawal reflex occurred, then anesthesia was not sufficient. Laminectomy was performed to expose the lumbar spinal cord (L3-L6). The animal was then placed in a rigid spinal unit frame, and the dura mater covering the spinal cord was dissected. The skin flaps were used to form a pool that was filled with warm paraffin oil.
Surface potentials were recorded with bipolar Ag-AgCl ball electrodes with an active lead placed on the surface of the dorsal column of the spinal cord and a reference lead placed in the nearby connective tissue. The recording was made from the lumbar spinal cord (L4 and L5) because this is the area where the maximal evoked potential from stimulation of the hindlimb foot pad could occur. Capsaicin was applied acutely to investigate the contribution of C-fibers on spinal cord evoked potentials.

2.9 Acute preparation for cortical recording

The anesthesia procedure was the same as the spinal cord recording experiment. When the paralysis test was required, animals were immobilized with gallamine triethiodide (Flaxedil, 40 mg/kg, i.v.) and then artificially ventilated. End tidal CO_2 concentration was monitored and maintained at approximately 3-4%. Heart rate, respiratory rate, and pCO_2 were monitored throughout the recording session. Six rats were allowed to periodically recover from muscle paralysis to assess the arousal level of anesthesia by evaluating the corneal reflex or withdrawal reflex of the hindlimb. Supplementary doses of ketamine (10 mg/kg) were administered if reflexes were present.

2.10 Data analysis

The amplitudes of the laser-evoked cortical potentials and spinal cord potentials and the total frequency of each response over the 20 trials were obtained for each rat. Analysis of variance (ANOVA) was used to assess the effects of capsaicin treatment.

3. Results

3.1 Effects of capsaicin on spinal cord and cortical potentials evoked by mechanical and laser stimulation

Mechanical stimulation of the hindpaw evoked a short negative potential followed by a long-lasting positive potential in the dorsal lumbar column surface. Applying capsaicin to the sciatic nerve did not influence this potential (Fig. 2A, left panel). In contrast, a short laser pulse (10 W, 30 ms) applied to the hindpaw induced a prominent positive potential in the dorsal column of the lumbar spinal cord (L4-L5) (Fig. 2A, lower right panel). The LEP amplitude in the spinal cord was significantly lower in the capsaicin group (Fig. 2A, upper right panel) compared with the vehicle group, indicating that capsaicin specifically blocked the transmission of sensory information from the peripheral nerve to the spinal cord.

3.2 LEP conduction velocity

To estimate the conduction velocity of LEP in peripheral transmission, we recorded field potentials in the spinal cord evoked by laser pulses applied to two sites, 10 mm apart, on the hindpaw (Fig. 2B). To calculate the conduction velocity, the difference in the latency of both LEPs produced by stimulating the proximal and distal paw regions was divided by the distance (10 mm) between the sites. The LEP conduction velocity was 0.8 ± 0.3 m/s (n = 5), which was in the range of C-fiber conduction. The latency for spinal cord evoked potentials was in the range of 70 to 90 ms, which was close to the latency of the laser-evoked EMG response of the hindlimb. Thus, the EMG response recorded in the hindlimb was likely induced by C-fiber activation. The latency of cortical LEPs had a much longer latency than both the EMG responses and the spinal cord evoked potentials. Thus, the ascending spinal pathway appears to convey such nociceptive information to the supraspinal brain areas.

3.3 Laser-evoked cortical potentials

Short-pulse, low-intensity, CO_2 laser irradiation of the hindpaw in awake, freely moving rats produced distinct evoked cortical potentials in the S1 region. Prominent negative potentials were evoked and could be distinguished from background potentials. The peak latency of the LEP was 254.2 ± 11.9 ms, and the amplitude was -0.16 ± 0.05 mV. Laser-evoked potential components could be induced bilaterally, and their amplitudes increased as laser intensity increased (Fig. 3A, B). An earlier component was discernible at a latency of approximately 60 ms, but because of the variation in amplitudes and nonsignificant influence of capsaicin, this early component was not analyzed further.

3.4 Component profiles of nocifensive behavioral responses

Consistent with our previous findings [Fan et al., 1995], we found that as stimulus intensity increased, animals exhibited a greater variation in responses and a greater frequency of component responses (Fig. 3C). When the hindpaw was stimulated with 5 W laser power, the rats reacted with few withdrawal, flinch, and body movement responses. At 10 W, their

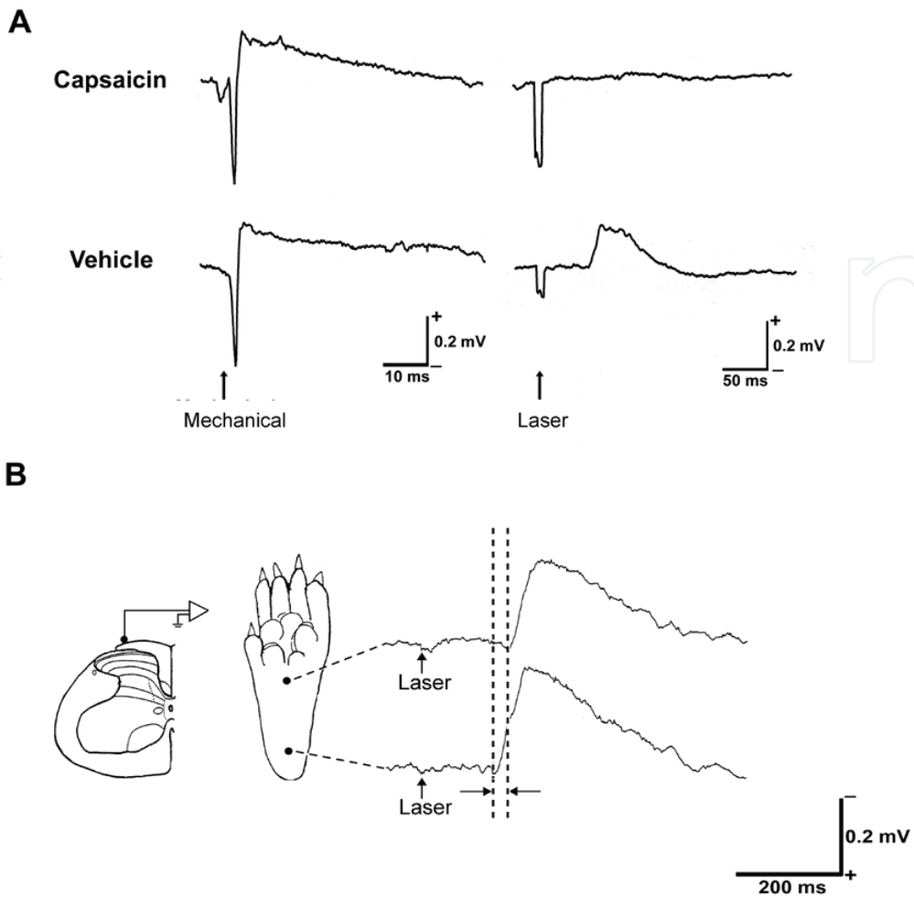

Fig. 2. Spinal cord evoked potentials and conduction velocity measurement. **A.** Spinal cord evoked potentials induced by either mechanical or CO_2 laser stimulation. Applying capsaicin to the left sciatic nerve in rats affected spinal cord LEPs but not mechanical-evoked potentials. No changes were found in vehicle group potentials evoked by either of the stimulation methods. **B.** Conduction velocity of spinal cord potentials evoked by CO_2 laser stimuli. The recording site was located in the dorsal column of the spinal cord. Laser stimulation was applied to the heel and middle regions of the hind paw. The representative evoked potential for each stimulated point is shown in the right panel. The difference in the response latency for both stimulation points was calculated and divided by the total distance from the hindpaw to the spinal cord.

withdrawal/flinch response frequency increased by more than 50%, with very few body movements and licking. Behavioral responses markedly changed when stimulated with 20 W, with withdrawal/flinch, body movement, and lick responses increasing by 40%, 30%, and 70%, respectively. At 25 W, a greater number of lick responses and 20% and 50% more withdrawal/flinch and body movement responses, respectively, were evident.

The of withdrawal/flinch, body movement, and lick response components corresponded to the cortical LEP amplitude (Fig. 3D). At 3 W laser power, LEPs were less than 0.1 mV. At 5 W, the rats reacted with withdrawal/flinch responses which produced ~0.2 mV of cortical LEPs. The amplitude increased to ~3-4 mV when body movements and licking were elicited by stimulation at the 10 and 20 W stimulus intensities.

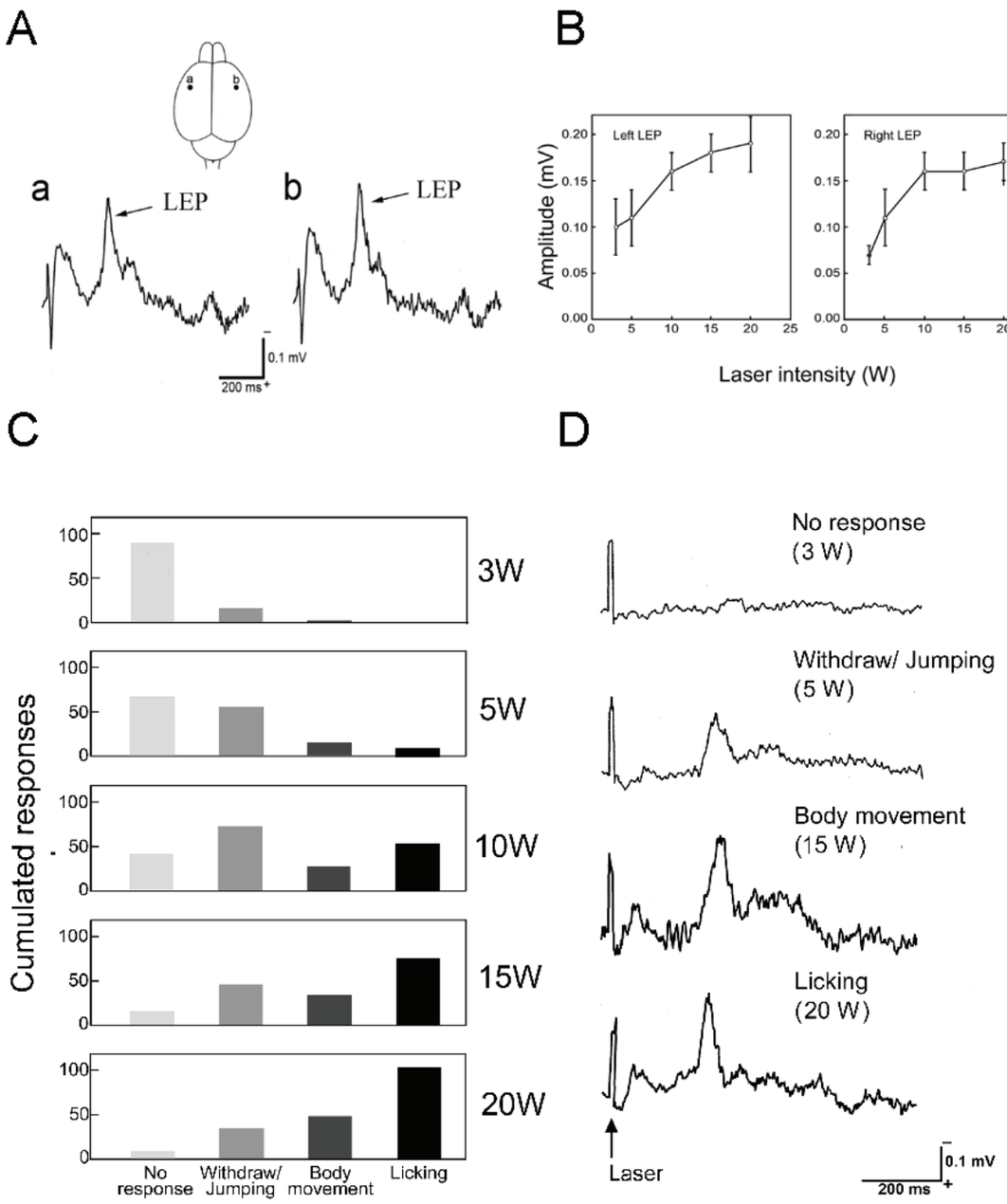

Fig. 3. Typical examples of cortical field potentials evoked by noxious laser pulses and nocifensive behavioral response component profile. **A.** Laser-evoked potentials were bilaterally recorded in the cortex. **B.** The graphs show the mean amplitude of LEPs recorded from the right or left side as a function of laser intensity. **C.** Average response frequency for each behavioral response component at different laser stimulation intensities ranging from 5 to 25 W. **D.** The representative evoked potentials corresponded to different behavioral elements: no response, withdrawal/flinch, body movement, and licking.

3.5 Effect of capsaicin on nocifensive behavioral components

Capsaicin treatment did not noticeably alter locomotion or the righting response during handling or open field exploration. Furthermore, capsaicin and vehicle treatment did not produce trophic or morphological changes in the lower extremities.

Laser stimulation of the footpad in freely moving rats induced several behavioral components, such as flinching, withdrawing, licking, moving, and head turning. The occurrence of some behavioral components were markedly altered after capsaicin treatment. The response frequency for flinching, withdrawing, and licking were significantly reduced after stimulation on the capsaicin-treated side, whereas the frequency for moving and head turning were not affected. Additionally, the reduced incidence of behavioral components after capsaicin treatment did not recover for at least 14 days.

Rats showed an average of three flinch responses induced by laser stimulation (10 W, 30 ms) before capsaicin treatment. After capsaicin treatment, this response decreased to an average of less than one time. This observation was confirmed statistically using a *post hoc* test, in which the flinch response significantly decreased compared with the vehicle treatment group ($F = 4.41$, $p < 0.05$) on days 3, 5, and 14 after treatment. Similarly, withdrawal responses decreased after capsaicin treatment. Before treatment, rats exhibited the withdrawal response 5-6 times, but following treatment, the capsaicin-treated group responded less compared with the untreated side and vehicle group ($F = 13.03$, $p < 0.001$) on days 3, 5, and 14 ($p < 0.01$) after treatment. After the laser pulse stimulation was applied to the hindpaws, the frequency of the licking responses was also reduced from 2-4 times before capsaicin treatment to approximately 1 time ($F = 6.56$, $p < 0.01$) on Days 3, 5, and 14 after treatment. In contrast to the above behaviors, rats only moved or turned their heads 1-2 times before treatment, and this frequency did not change after capsaicin treatment.

Typical effects of capsaicin and vehicle treatment on LEPs in behaving rats are shown in Fig. 4. Laser-evoked potentials were markedly diminished after the left sciatic nerve was treated with capsaicin but not with vehicle (Fig. 4A, B, and right panel). A prominent fast component could be elicited by mechanical stimulation, and this component was unaltered after capsaicin treatment. The amplitude of LEPs decreased by an average of 78% in the capsaicin group compared with the vehicle group and untreated paws of both groups. These observations were confirmed statistically. The amplitude of cortical LEPs produced by stimulating the left paw was significantly reduced in the capsaicin-treated group compared with the vehicle group ($F = 4.95$, $p < 0.05$; Fig. 4C). However, no significant differences were found for mechanical-evoked cortical potentials (Fig. 4D).

4. Discussion

This study showed that capsaicin, which selectively blocks activity of primary sensory C-fibers, inhibited specific components of nocifensive behavior. Applying capsaicin to the sciatic nerves of rats blocked flinch, withdrawal, and lick responses but did not affect movement and head turn responses. These results suggest that C-fibers mediate the nocifensive components of flinching, withdrawing, and licking. Interestingly, these components were temporally linked to activation of the nocifensive motor system and cortical-evoked potentials.

Nocifensive behaviors elicited by CO_2 laser stimulation were linked to LEPs recorded in freely moving rats. The average latency of late LEPs was 250 ms, which is in the range of C-

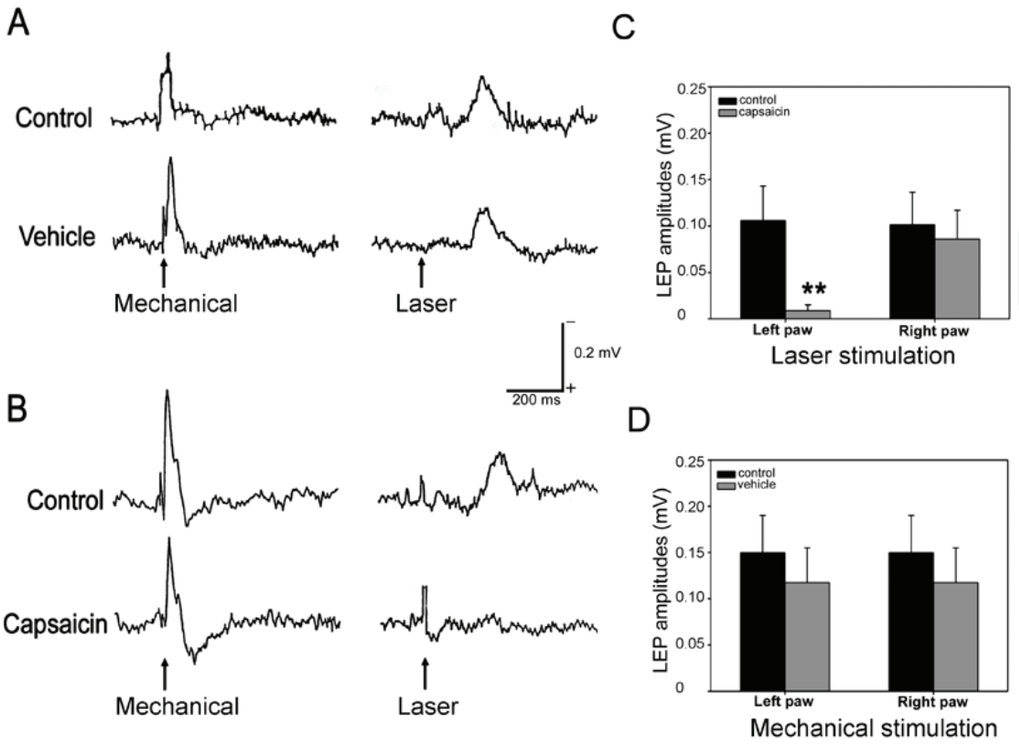

Fig. 4. Effect of capsaicin on cortical potentials evoked by mechanical and laser stimulation. The sciatic nerve of the left leg of rats was treated with capsaicin or vehicle (n = 6/group), whereas the right leg was untreated (Control). **A, B.** Traces of recorded potentials for the control, vehicle, and capsaicin groups. Mechanical and laser stimulation was applied to the right (A) and left (B) paws. **C, D.** Statistical analyses showed that the LEP (C) was significantly reduced in the capsaicin group compared with the untreated paw and vehicle group, but not with mechanical stimulation (D). $^{**}p < 0.01$.

fiber conduction [Bromm & Treede, 1987]. This result is closely related to C-fiber-mediated cortical responses evoked by laser pulses in anesthetized [Shaw et al., 2001] and awake, freely moving rats [Shaw et al., 1999]. Capsaicin, a neurotoxin known to block C-fiber activity [Wall & Fitzgerald, 1981], decreased the frequency of nocifensive responses, the amplitude of spinal evoked potentials, and LEPs when applied to the sciatic nerves of rats. Altogether, these results suggest that the nocifensive response components, including flinching, withdrawing, and licking, were mediated by C-fibers via the spinal cord and then linked to neurons in the cortex, which was demonstrated by LEP induction. We recently analyzed the current source density of LEPs in the S1 region of the cortex and found that intracortical current flows revealed late synaptic activation that initially occurred in layer VI and were relayed transsynaptically to both superficial and deep layers [Sun et al., 2006].

Evaluating the respective contributions of both Aδ- and C-fibers for a given test of nociception is challenging. Capsaicin administration primarily destroys C-fibers in rats and sometimes certain Aδ-fibers, albeit to a lesser extent [Lynn, 1990]. To exclude the possibility

of Aδ-fiber destruction, we applied electrical stimulation on treated and untreated foot paws. Subsequently, we found that the animals had evoked spinal and cortical potentials. The latency of the evoked potentials was in the range of 60 ms, and their amplitudes were significantly higher than that of the capsaicin-treated group. These results demonstrate that faster conduction fibers, in the range of Aδ-fibers, were not destroyed. Therefore, we are confident that the behavioral components, including flinching, withdrawing, and licking, are nocifensive responses mediated by C-, but not Aδ-, fibers. In experiments conducted in humans, in which a brief and sufficiently intense single stimulus activates Aδ- and C-fibers, the emotional component of the secondary pain is much stronger than the primary pain [Handwerker & Kobal, 1993]. In the future, we suggest using an alternative experimental design that combines this pain-conditioning model with the current behavioral model to distinguish sensory components from affective components [Kung et al., 2003, Shyu et al., 2003].

Ideally, an animal model of behavior related to nociception should possess the following characteristics: (*i*) nociceptive stimulation ("input specificity"), (*ii*) a distinction between nociceptive and non-nociceptive stimuli, (*iii*) quantifiable responses that are correlated to stimulus intensity within a reasonable range (from thresholds for pain to pain tolerance), (*iv*) sensitivity to manipulations (notably pharmacological), which would specifically reduce nociceptive behavior, (*v*) differentiation between nonspecific behavioral changes (e.g., motility and attention) from those triggered by the nociceptive stimulus *per se*, (*vi*) consistent scores between identical or equivalent retests, and (*vii*) reproducible results. Most, if not all, of these characteristics are present in the model used in the present study. The behavioral model used here involved the use of a noxious stimulus, CO_2 laser, to elicit specific nocifensive response components. These nocifensive components (such as flinching, withdrawing, and licking) could be quantified, manipulated by capsaicin, and distinguished from non-nocifensive behaviors (e.g., head turning and moving). Moreover, these response components were consistently elicited when stimulated with CO_2 lasers and are reproducible because they have been described in reports from other laboratories [Shaw et al., 1999].

A laser pulse radiates intense and highly focused thermal energy and has been used as noxious stimulation in several human studies [Arendt-Nielsen & Bjerring, 1993, Kakigi et al., 2005]. Carmon et al. reported that an energy density of 1.64 to 1.94 J/cm ^{2} caused a slight burning pain sensation in subjects, and an energy density of 2.49 to 2.54 J/cm^2 caused a moderate pinprick pain sensation [Carmon et al., 1976]. In the present study, we used the same laser stimulus source and found that the energy densities that led to lick and foot movement responses were 1.93 and 3.11 J/cm^2, respectively. Thus, the range of energy density values obtained in the present animal study is comparable to that from human studies. In humans, when the stimulus intensity was increased to achieve progressive levels of pain, the amplitude of evoked cortical responses also increased, and the correlation between cortical responses and weighted subjective responses was significant (r = 0.707) [Carmon et al., 1976]. In the present study, we found that various intensities of CO_2 laser stimulation applied to the same location in anesthetized and freely moving rats induced dorsal horn activity and two cortical potentials, one with a latency of approximately 64 ms and a second with a latency of 253 ms. The spinal potential and the second cortical potential were sensitive to the stimulus intensity and susceptible to modification by capsaicin. These

data further suggest that our laser stimuli were indeed noxious and specific to certain nocifensive behavioral elements and neuronal activity in rats.

The three response components of the nocifensive behavior model were most likely mediated by C-fibers, and we suggest that the present model is suitable for studying the neuronal mechanisms underlying the analgesic effects of morphine. Morphine reduces the responses of dorsal horn neurons produced by C-fibers more easily than it affects those produced by Aδ-fibers [Jurna & Heinz, 1979]. This observation may explain why experimental pain in humans, which is usually produced by Aδ-fibers, is little affected by morphine [Becher, 1957]. Some behavioral models using phasic stimulation methods predispose human subjects and animals to respond to pain as soon as it occurs (i.e., at the moment the first pain is produced by Aδ-fibers). The presence or absence of secondary pain will generally have no impact on the measurement. For example, animals withdrew their hindpaw after high-intensity electrical stimulation [Evans, 1961]. This test may involve the activation of both Aδ- and C-fibers, as well as some non-nociceptive fibers. Stimulation is stopped as soon as a response is observed. Yeomans and Proudfit [1996] suggested that most common nociceptive tests involving mechanical and thermal stimuli actually investigate only responses triggered by Aδ-fibers and thus are not sensitive to morphine, with the exception of very high doses. In a pain-induced audible and ultrasonic vocalization experiment in rats, a vocal response was clearly triggered by C-fibers and was very sensitive to morphine, with an ED_{50} five-fold less than when it is triggered by Aδ-fibers [Jourdan et al., 1998]. We therefore propose that our nocifensive behavioral model is suitable for studying the dynamic analgesic effects of morphine.

In conclusion, the present results suggest that nocifensive behavior has distinct components that can be analyzed, and the reaction pattern changes probabilistically, such that the greater the noxious stimulation, the more likely additional components will be evoked [Fan et al., 1995]. The nocifensive motor system may be viewed as a set of hierarchically organized responses, and a given subset of responses appear with a specific noxious stimulation, dependent on stimulus intensity. The study of the mechanism of pain must consider this pain response hierarchy to precisely define the neurological bases of sensory and motor aspects of the nociceptive system.

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