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INFLUENCES OF SURGICAL DECOMPRESSION ON THE DORSAL HORN AFTER CHRONIC CONSTRICTION INJURY: CHANGES IN PEPTIDERGIC AND δ-OPIOID RECEPTOR (+) NERVE TERMINALS

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Abstract—To understand plastic changes in the dorsal horn related to neuropathic pain, we developed a model of decompression in rats with chronic constriction injury (CCI) and investigated corresponding changes in the dorsal horn. At postoperative week 4 (POW 4) of CCI, rats were divided into a decompression group, in which ligatures were removed, and a CCI group, in which ligatures remained. Spinal cords were immunostained for substance P (SP), the δ-opioid receptor (DOR), and calcitonin gene-related peptide (CGRP). Areas of immunoreactive nerve terminals in the dorsal horn were quantified and expressed as the dorsal horn index (immunoreactive areas of the operated side compared with those of the contralateral side). At POW 4, dorsal horn indexes of all of these molecules were significantly reduced in both groups to similar degrees (0.36∼0.43). At POW 8, neuropathic pain behaviors had completely disappeared in the decompression group with significant reversal of the dorsal horn indexes compared with the CCI group (0.81±0.02 vs. 0.58±0.09, $P<0.001$ for SP and 0.75±0.04 vs. 0.55±0.03, $P<0.001$ for DOR). In the CCI group, neuropathic pain behaviors became normalized at POW 12 with corresponding changes in dorsal horn indexes for both SP and DOR similar to those of the decompression group. In contrast, changes in the dorsal horn indexes of CGRP were similar in both the CCI and decompression groups throughout the experimental period. These findings suggest that CCI and decompression cause different patterns in peptidergic and DOR (+) nerve terminals in the dorsal horn.

Key words: nerve injury, substance P, neuropeptides, opioid receptor, neuropathic pain, calcitonin gene-related peptide.

Nerve injury induces a series of plastic changes in the central processes of dorsal root ganglion (DRG) neurons terminating in the dorsal horn, which reflect dysregulated protein synthesis and trafficking (Bailey and Ribeiro-da-Silva, 2006; Balasubramanyan et al., 2006; Torsney and MacDermott, 2006). The dorsal horn is the first relay center for transmitting nociceptive information; thus the central terminals of DRG neurons have profound effects on the development of neuropathic pain (Ma and Eisenach, 2003; Ro et al., 2004), for example, by reducing the content of substance P (SP) in the dorsal horn after chronic constriction injury (CCI) (Cameron et al., 1997; Goff et al., 1998), but not of the calcitonin gene-related peptide (CGRP) (Cameron et al., 1997; Li et al., 2004).

Compression-related nerve injury is the main model for neuropathic pain, and it includes CCI, partial sciatic nerve ligation, and spinal nerve ligation (Bennett and Xie, 1988; Seltzer et al., 1990; Kim and Chung, 1992). In clinical practice, surgical decompression is frequently used to relieve symptoms of neuropathic pain, e.g. carpal tunnel syndrome, spinal root compression, and trigeminal neuralgia due to vascular compression (Love and Coakham, 2001; Binder et al., 2002; Steinberg, 2002; Thoma et al., 2004). In addition to facilitating nerve regeneration, plasticity of the CNS, particularly, the dorsal horn of the spinal cord may play an important role in the reversal of neuropathic pain (Woolf et al., 1998; Woolf, 2000, 2004; Suzuki and Dickenson, 2005; Woolf and Salter, 2006). Taken together, these studies raise several intriguing issues; for example, whether such changes in the dorsal horn can be reversed if neuropathic pain disappears and whether different molecules in the dorsal horn follow the same patterns of change after decompression. There has been lack of animal systems investigating decompression and its effects on the temporal course of neuropathic pain behaviors and the above-described changes in the dorsal horn.

A recent observation indicated that trafficking of the δ-opioid receptor (DOR) was linked to release of SP (Guan et al., 2005; Julius and Basbaum, 2005). It remains elusive whether expression of the DOR shows the same trend as that of SP in neuropathic pain models, and whether such changes can be modulated by decompression. These molecular signatures may reflect or underlie the neuroplasticity of the dorsal horn related to the development neuropathic pain after nerve injury.

To address these issues, we established a model of decompression by removing all ligatures of CCI 4 weeks after neuropathic pain behaviors had been well established. Our study indicated that decompression speeded

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Abbreviations: CCI, chronic constriction injury; CGRP, calcitonin gene-related peptide; DOR, δ-opioid receptor; DRG, dorsal root ganglion; FITC, fluorescein isothiocyanate; IASP, International Association for the Study of Pain; IgG, immunoglobulin G; NK1, neurokinin 1; PB, phosphate buffer; POW, postoperative week; SP, substance P.

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up the disappearance of neuropathic pain behaviors with significant reorganization of central terminals in the dorsal horn.

EXPERIMENTAL PROCEDURES

Study design and surgical procedures

Adult male Sprague–Dawley rats, weighing 250~300 g, were used in these experiments. Three animals were housed together in plastic cages with soft sawdust as bedding to avoid mechanical damage to the hind-paw skin. These animals were placed in a temperature- and humidity-controlled room with a 12-h light/dark cycle. Food and water were available *ad libitum*. All procedures to minimize the number and suffering of animals were conducted in accordance with ethical guidelines set up by the International Association for the Study of Pain (IASP) on the use of laboratory animals in experimental research (IASP Committee, 1980; Zimmermann, 1983), and the protocol was approved by the Animal Committee of National Taiwan University College of Medicine, Taipei, Taiwan.

CCI was induced in animals following established surgical procedures (Bennett and Xie, 1988; Lin et al., 2001). Briefly, under chloral hydrate anesthesia (400 mg/kg, i.p., Sigma Chemicals, St. Louis, MO, USA), the right sciatic nerve was exposed at the mid-thigh level by freeing the adhering fascia between the gluteus and biceps femoris muscles. Four ligatures (of 4/0 chromic gut, Ethicon Inc., Somerville, NJ, USA) were loosely tied around the sciatic nerve at 1-mm intervals above the nerve's trifurcation. Ligatures constricted only about 1/3~1/4 of the diameter of the nerve and produced a small, brief twitch in the muscle around the exposure. The circulation through the superficial epineural vasculature was blocked between the ligatures. This side was defined as the operated side; the contralateral side was used for comparison to normalize individual variations of different animals.

To examine the effect of decompression on neuropathic pain and nerve regeneration, animals were randomly assigned to two groups. In one group, all four ligatures were carefully removed without destroying the surrounding vessels at postoperative week 4 (POW 4); this group was designated the decompression group. As we reported before, ligatures were visible although reactive fibrosis became prominent at POW 4; under a dissecting microscope, ligatures could be untied without destroying the surrounding tissues (Tseng et al., 2007b). The other group was designated the CCI group, in which ligatures remained throughout the experimental period. Examiners were blinded to the grouping information, and this information was only decoded during the final analyses. To exclude the influences of surgical procedures, we added two control groups: (1) a decompression-control group (i.e. re-exposure of the sciatic nerve with dissection of surrounding connective tissues but without actual removal of sutures) and (2) a negative-control group (i.e. surgical mobilization of the contralateral nerve).

Thermal hyperalgesia

We evaluated thermal hyperalgesia with a Hargreaves-type analgesiometer (Ugo Basile, Comerio-Varese, Italy) by measuring the paw withdrawal latency upon heat stimulation. Rats were individually placed in one of three separate Plexiglas containers (22×17×14 cm) located on an elevated floor of a clear glass plate (3 mm thick) and allowed 30 min to habituate to the apparatus. A radiant heat source was placed directly beneath the plantar surface of the hind paw. The withdrawal latency was automatically measured as the time elapsed from the onset of radiant heat stimulation to the withdrawal of the hind paw. A maximal time of 20 s for the thermal stimulus was imposed to avoid possible tissue damage. Each hind paw was alternatively tested seven times with a minimal interval of 5 min between measurements, and readings were recorded to the nearest 0.1 s. Values of the last five consecutive measurements were used for the analysis (Chiang et al., 2005).

Mechanical allodynia

Mechanosensitivity was determined by measuring the withdrawal thresholds to a series of calibrated von Frey filaments (Senselab aesthesiometer, Somedic Sales AB, Stockholm, Sweden) according to the up-and-down method (Chiang et al., 2005). Rats were individually placed in one of three separate Plexiglas containers on a wire mesh floor and allowed to acclimate for 10 min. The examiner touched the plantar surface of the hind paw with a filament for 1 s until the bending angle reached 45° with a brisk withdrawal or paw flinching was noted, which was considered a positive response. Five stimuli using the selected hair were applied at 5-s intervals. If there was no withdrawal response to the initially selected hair with these five stimuli, a stronger stimulus was applied. If the animal withdrew its hind paw in response to any of the five stimuli, the next weaker stimulus was chosen. The mechanical threshold was defined as the minimal force (g) initiating a withdrawal response.

Immunohistochemistry of spinal cords

Animals were killed by an intracardiac perfusion of 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. Spinal cords (lumbar segments 4 and 5) were fixed for another 6 h and then changed to 0.1 M PB for storage. After a thorough rinsing in PB, samples were cryoprotected with 30% sucrose in 0.1 M PB overnight. Sections (50-μm cross-sections of the spinal cord) were cut on a sliding microtome (HM440E; Microm, Walldorf, Germany), labeled sequentially, and stored at −20 °C. To ensure adequate and systematic sampling, every sixth section of the spinal cord was immunostained (Lin et al., 2001). Sections were treated with 0.5% Triton X-100 in 0.5 M Tris buffer (Tris), pH 7.6, for 30 min and processed for immunostaining. Briefly, sections were quenched with 1% H_2O_2 in methanol and blocked with 5% normal goat serum. Sections were incubated with primary antiserum overnight. After rinsing in Tris, sections were incubated with biotinylated goat anti-rabbit IgG for 1 h and the avidin–biotin complex horseradish peroxidase reagent (Vector Laboratories, Burlingame, CA, USA) for another hour. Reaction products were demonstrated with 3,3′-diaminobenzidine (DAB, Sigma Chemicals).

Antibody characterization

The polyclonal anti-SP antibody (1:1000, catalog no. 20064, lot no. 313003, DiaSorin, Stillwater, MN, USA) was raised in rabbit against SP coupled to keyhole limpet hemocyanin (KLH) (Ruscheweyh et al., 2007). The guinea-pig anti-SP antibody (1:1000, catalog no. T-5019, lot no. 021077–4, Peninsula Laboratories, Belmont, CA, USA) was raised against a partial sequence of the human SP (H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) (Takeuchi et al., 2007). The immunogen of the rabbit anti-CGRP antiserum (1:1000, catalog no. AB1971, lot no. 24040375, Chemicon, Temecula, CA, USA) was a synthetic rat α-CGRP conjugated to bovine serum albumin (Yen et al., 2006). All of these antisera were characterized and compared with previously published articles regarding the staining patterns in the spinal cord, DRG, and cutaneous structures for SP (Ruscheweyh et al., 2007; Takeuchi et al., 2007) and CGRP (Hsu et al., 2004; Dobolyi et al., 2005; Yen et al., 2006). The anti-DOR antiserum (1:1000, RA10100, lot no. 400012, Neuromics, Minneapolis, MN, USA) was raised in rabbits against a synthetic peptide at the N-terminus of mouse DOR (LVPSARAELQSSPLV, i.e. amino acids 3~17) and reacted with an appropriate molecular weight on Western blotting (Arvidsson et al., 1995; Cahill et al., 2001). The specific-