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Inflammatory hypersensitivity in a rat model of trigeminal neuropathic pain

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KEYWORDS
Trigeminal; Neuropathic pain; Hyperalgesia; Inflammation; Rat; Infraorbital nerve

Summary Thermal and mechanical stimuli have been used to monitor the development of neuropathic pain following an experimental injury to a branch of the trigeminal nerve. However, the response to inflammatory challenge has not been evaluated in a model of orofacial neuropathic pain. The purpose of this study was to determine whether chronic constriction of the infraorbital nerve (IoN) enhances nociceptive responses elicited by the formalin test. The characteristic biphasic response (primarily directed grooming) to formalin injected subcutaneously in the right vibrissae pad was observed in sham-injury rats. Twenty-one days after IoN constriction, formalin injection provoked an immediate response that involved both directed grooming and other abnormal behaviors, e.g. flinching, trismus and shielding of the affected region. As with sham-injury rats, this was followed by a quiescent period and then a second phase of nocifensive behaviors. The total time recorded for all pain-related behaviors was significantly greater in rats with constrictive injuries ($P < 0.001$), due primarily to the exhibition of novel pain-related responses. Histological examination (qualitative) revealed that chronic constriction resulted in a ligature-induced neuroma, as well as a partial denervation of the affected sensory field. Thus, an intense inflammatory hypersensitivity in a rat model of orofacial neuropathic pain develops in association with partial denervation and with an ongoing perineural inflammatory response and neuroma formation.

Introduction

Experimental injury (constriction, partial ligation) of the sciatic nerve or ligation of the L5/L6 spinal nerves leads to the development of neuropathic pain behaviors in rats,[1–5] and these models have been used to study the basic mechanisms underlying the development of mechanical allodynia and thermal hyperalgesia after injury to a spinal nerve.[6] Chronic constriction of a trigeminal nerve also has behavioral, physiological and neuroanatomical outcomes, many of which are similar to those observed after spinal nerve injury.[7,8] For example, mechanical allodynia and thermal hyperalgesia can be demonstrated following chronic constriction of the infraorbital nerve (IoN). However, the evidence also suggests that the consequences of inferior alveolar and IoN injuries differ from those seen after injury to the sciatic nerve.[8]

Although our understanding of the effects of nerve injury on mechanical and thermal allodynia and hyperalgesia has dramatically increased, very little is known about the effects of such injuries on the behavioral responses to inflammatory or chemogenic challenge. Recently, LaBuda et al.[5] were the first to report that L5 spinal nerve ligation

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results in an enhanced nociceptive response to formalin injection in the hind paw of the rat, and they pointed to the potential significance of coexisting neuropathic and inflammatory conditions in the clinical management of pain.

Inflammation per se results in hyperalgesia[9–11] and one common protocol used to examine this phenomenon has been the formalin test.[12] This test was adapted for the study of orofacial pain mechanisms,[13–18] and a consistent pattern of nocifensive behaviors has emerged. Following an initial nociceptive response (directed grooming), which is associated with the chemogenic stimulation of high-threshold A_δ and C fibers, there is a quiescent period. As the inflammatory response develops, increases in peripheral nociceptive fiber sensitivity (peripheral sensitization to inflammatory mediators, such as bradykinin), and the facilitation of neuronal activity in the dorsal horn of the spinal cord (central sensitization due to increased peripheral input) lead to a second period of nocifensive behaviors. This latter phase builds and then gradually subsides over a period of 20–40 min.

Neuropathic pain syndromes, such as post-herpetic neuralgia and trigeminal neuralgia (both idiopathic and secondary), affect the orofacial region, but to our knowledge the response to inflammatory pain had not been examined in an animal model of trigeminal neuropathic pain. Thus, the purpose of this study was to measure the effects of chronic constriction of the IoN on the severity of inflammatory pain evoked during the formalin test. Mechanical allodynia has been consistently demonstrated in this model,[19–24] but this is the first report of inflammatory hypersensitivity following an experimental trigeminal neuropathic injury.

Methods

Twenty-four male, Wistar rats (Simonsen Laboratories, Gilmore, CA) initially weighing 225–250 g were housed three to a cage for 7–10 days prior to the beginning of the study. Animals were kept in a temperature and light controlled environment (12:12 h, light:dark), and allowed free access to standard laboratory chow and water. All housing conditions and experimental procedures conformed to protocols approved by the University of the Pacific Animal Use Committee, and were consistent with the ethical guidelines published by the International Association for the Study of Pain.

In 16 rats, a chronic constrictive injury (CCI) of the right IoN was performed under pentobarbital anesthesia (50 mg/kg i.p.) via an intraoral approach.[20] Briefly, a 1 cm long incision was made in the buccal mucosa, and the IoN was freed from surrounding muscle and connective tissue. A single 5–0 chromic gut ligature was then placed loosely around the branches of the IoN as it emerged from the infraorbital groove. The ligature was then positioned as close to the infraorbital groove as possible. The wound was checked for hemostasis and the incision was closed using three 6–0 silk sutures. Sham injuries (exposure of the IoN) were performed in another eight animals, and care was taken not to stretch the nerve or damage the epineurium. No antibiotics were administered, and all rats recovered uneventfully.

Twenty-one days after surgery, CCI and sham-injury rats were acclimated to the testing room for at least 30 min, and then placed in a clear acrylic cage (25 cm × 45 cm × 20 cm) without bedding for an additional 20 min. A 30 gauge needle was used to subcutaneously inject 50 μl of 3% formalin in saline into the ipsilateral upper lip (region of vibrissae C/D:3/4). In three CCI rats, the formalin injections were given into the contralateral upper lip, and as an additional control, three CCI rats received 50 μl of saline only into the ipsilateral vibrissae pad. The rats were immediately returned to the testing cage, and behavior was monitored for 45 min. At the end of each 45 min observation, the animals were placed in their normal cages and returned to the animal quarters.

Responses were scored as: (1) normal non-nociceptive behavior (e.g. sitting quietly or exploring); (2) directed grooming; or (3) other pain-related behaviors (e.g. flinching, trismus and shielding of the affected region). Previously published studies[13–18] utilized nocifensive grooming as the objective measure. However, preliminary trials to determine the appropriate formalin concentration revealed the second category of abnormal behaviors. The cumulative times spent in each type of behavior were recorded during 15 consecutive 3-min intervals with the aid of a computer program.

To evaluate the extent of nerve injury and cutaneous deafferentation after IoN constriction, two additional CCI rats (not used for behavioral testing) were anesthetized with Nembutal® (50 mg/kg i.p.) and tissues were fixed by transcardiac perfusion with 4% paraformaldehyde + 0.2% picric acid in 0.1 M PO_4 buffer, pH 7.4. Both the ipsilateral and contralateral infraorbital nerves were dissected out and placed in half-strength Karnovsky's fixative (2% paraformaldehyde + 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) for 4 h at 4 °C. For each injured nerve, the injury site (proximal and distal to the ligature) and 4–5 mm of nerve proximal to the constriction were harvested. Following fixation, the tissues were rinsed in buffer overnight

at 4 °C, post-fixed with OsO_4 and embedded in Epon. One micrometer sections were cut, mounted on glass slides and stained with methylene blue/azure II.

For evaluation of cutaneous innervation, skin biopsies from both the ipsilateral and contralateral IoN territories were fixed in paraformaldehyde/picric acid for an additional 4 h at 4 °C, rinsed with 0.1 M PO_4 buffer and cryoprotected by overnight immersion in buffer + 30% sucrose. Fifty micrometer sections were cut on a freezing microtome, and free-floating sections were processed for the immunocytochemical localization of PGP9.5, which is a general neuronal marker. After blocking endogenous peroxidase activity, the sections were incubated for 3 days at 4 °C with a rabbit anti-serum to PGP9.5 (1:4000, Chemicon International, Temecula, CA). After incubation with the primary antibody, the tissues were reacted with a biotinylated goat anti-rabbit IgG (1:10,000, Vector Laboratories, Burlingame, CA). Localization of PGP9.5 immunoreactivity was accomplished by the avidin–biotin technique (Elite® kit, Vector) and nickel-enhanced diaminobenzidine. The sections were mounted on glass slides and counter stained.

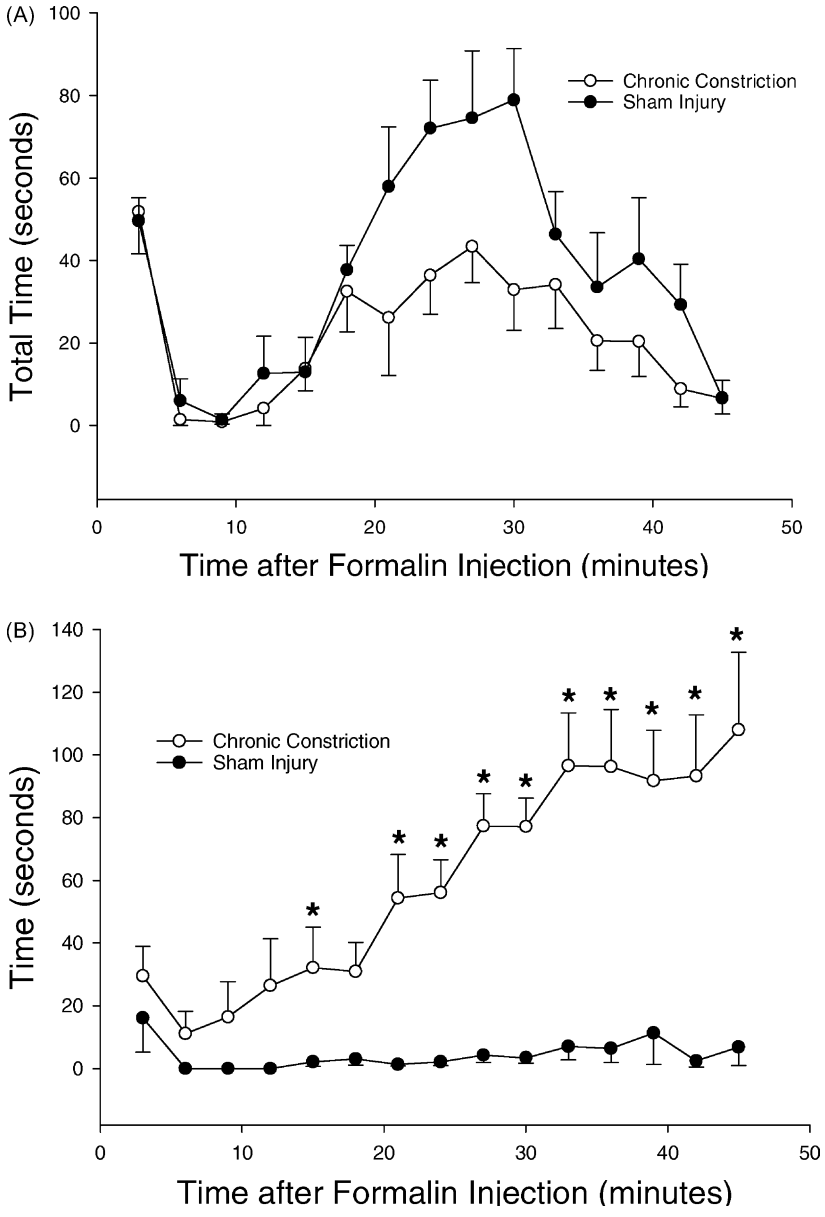

Figure 1 The time (s) spent in (A) directed grooming and (B) other pain-related behaviors during 15 consecutive 3-min periods following subcutaneous injection of 50 μl of 3% formalin into the upper lip. Each value represents the mean ± S.E.M. for eight CCI (M) and eight sham-injury (Φ) rats. $^{*}P < 0.05$ vs. sham-injury for a given 3-min interval. Note that no statistically significant differences were observed in directed grooming for individual time points.

Behavioral data were analyzed for statistical significance with SigmaStat 2.0 for Windows. A repeated measures ANOVA with injury as the between subjects variable, and test time period as the within subject factor was utilized for the analysis of group differences. Individual differences for each 3-min period were subsequently analyzed by the Student—Newman—Keuls' method for multiple comparisons. Differences in total response time and response latency were analyzed by Student's *t*-test. The α level for all tests was set at 0.05.

Results

Weight gain and general behavior after nerve injury

Neither sham-injury nor CCI rats demonstrated any obvious behavioral changes as a result of the surgical procedures, and food and water intake was unaffected. Initial body weights were similar for both sham-injury (227 ± 4 g, mean $\pm$ S.D.) and CCI (232 ± 4 g) rats. All animals demonstrated comparable weight gains, and the final body weights at the time of formalin testing were 313 ± 10 and 318 ± 11 g, respectively.

Nociceptive responses to formalin

The analysis of nociceptive responses in sham-injury and CCI rats revealed several significant differences between the two groups (Fig. 1). In sham-injury rats, a brief latency period after the injection of 3% formalin (49 ± 12 s, mean $\pm$ S.D.) was followed by the first phase of nocifensive behaviors (50 ± 16 s of directed grooming during the first 3 min). After a short quiescent period (from 3 to 15 min), a prolonged second phase of nocifensive behavior (beginning at 15—18 min) was characterized by an increase in directed grooming (504 ± 200 s) that gradually subsided over the remaining 30 min of the test. Other pain-related behaviors were only rarely recorded (72 ± 119 s total time). In contrast, CCI rats demonstrated an immediate response to formalin injection (latency $\sim$3 s, $P < 0.001$) that involved both directed grooming (52 ± 29 s) and other pain-related behaviors (30 ± 27 s). This was followed by a quiescent period, and then a second period of directed grooming (280 ± 123 s total duration) and other pain-related behaviors (857 ± 349 s total duration). Unlike the pattern for directed grooming, which gradually subsided over time, the frequency and duration of these other pain-related behaviors increased throughout the testing period.

The cumulative times spent in each category of behavior are shown in Fig. 2. Compared with sham-injury rats, CCI rats recorded significantly less time in normal, non-nocifensive behaviors ($P < 0.01$). Total time spent in directed grooming was also less for CCI rats ($P < 0.05$). However, total time for other pain-related behaviors was greater in CCI compared with sham-injury animals ($P < 0.001$). Thus, the total time recorded for all pain-related behaviors

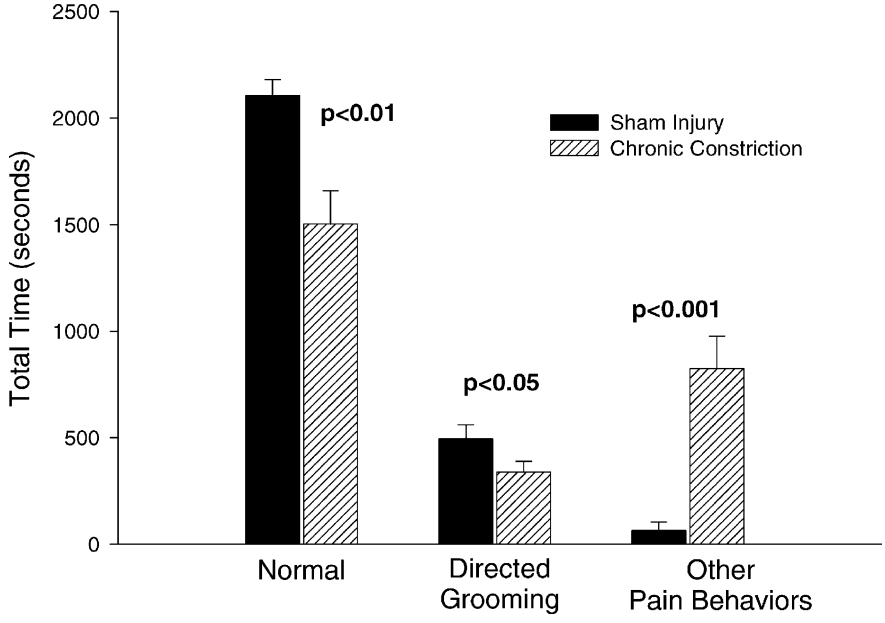

Figure 2 The cumulative time spent in normal, direct grooming and other pain-related behaviors during 45 min following the subcutaneous injection of 3% formalin (50 μl) into the upper lip. The bars represent the mean $\pm$ S.E.M. for eight CCI (hatched) and eight sham-injury (solid) rats.

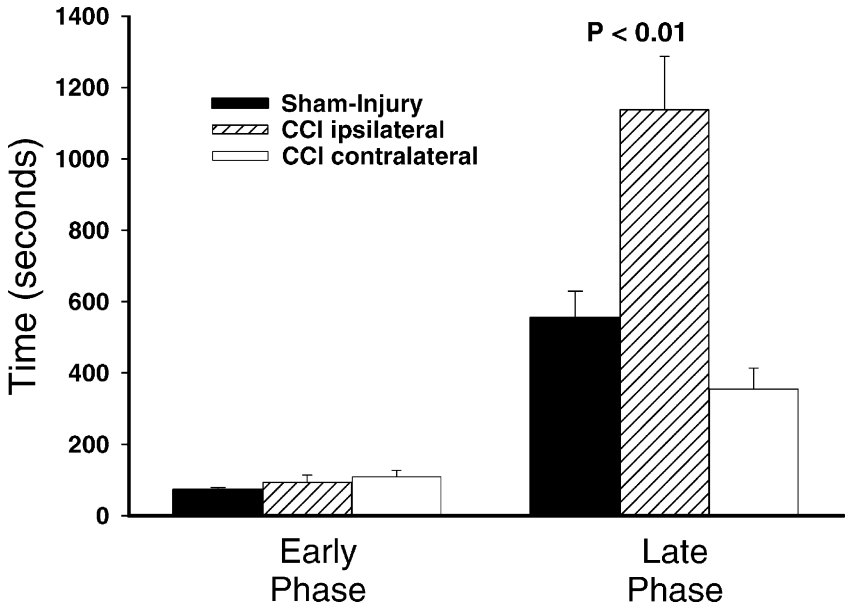

Figure 3 The total time spent in all pain-related behaviors during the early (0—6 min) and late (9—45 min) phases of the response to subcutaneous injection of 3% formalin (50 μl) into the upper lip. The values represent the mean ± S.E.M. for eight CCI (hatched bars) and eight sham-injury (solid bars) and eight rats. The response to a contralateral injection of formalin is shown by the open bars ($n = 3$). *P*-value, CCI vs. sham and ipsilateral.

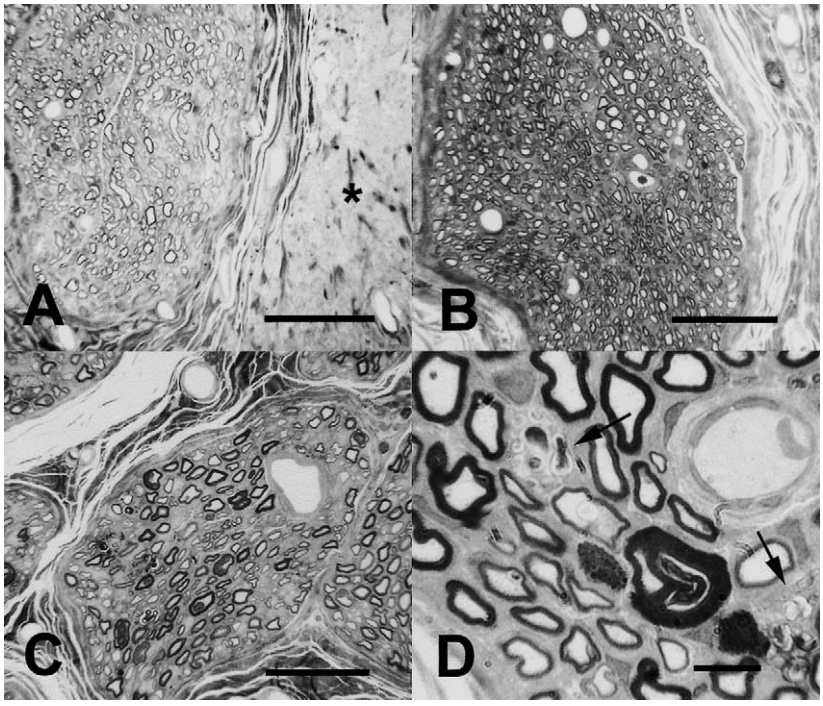

Figure 4 Representative 1 μm sections of infraorbital at the site of injury 21 days after chronic constriction. (A) Nerve fascicles immediately adjacent to the ligature contained numerous thin, lightly myelinated axons. Granulation tissue surrounding the ligature indicated by the asterisk. The bar represents 50 μm. (B) A nerve fascicle 1—2 mm distal to the ligature. Note the increased amount of endoneurial tissue around the predominantly thin, myelinated axons. The bar represents 50 μm. (C) This nerve fascicle is representative of those found near to, but outside of the chromic gut ligature. The amount of endoneurial tissue is greater than normal (see A). The bar represents 50 μm. (D) Another nerve fascicle from the same region as shown in (C), but at higher magnification (bar represents 10 μm). A degenerating, myelinated nerve fiber can been seen. Two mast cells are present, as are two macrophages (arrows) that contain large phagosomes. Mast cells were occasionally seen in uninjured nerves, as well.

was significantly greater after CCI (1231 ± 474 s) than after sham-injury (626 ± 218 s), $P < 0.001$. No pain-related behaviors were observed after saline injection, nor were differences observed between sham and CCI rats when formalin was injected contralateral to the injury site.

Nocifensive behaviors were also analyzed with respect to early (0—6 min) and late (9—45 min) phases. With the exception of the decrease in the latency of the initial response, no significant differences in early phase behaviors were observed between sham-injury and CCI rats (Figs. 1 and 3). However, a significant difference in total duration of nocifensive behaviors was measured between the two groups during the second phase ($P < 0.01$).

Anatomical evaluation of the infraorbital nerve

Gross observation of the injury site revealed the presence of a large granuloma (approximately 3—4 mm in diameter) surrounding the remnants of chromic gut, and a marked inflammatory cell infiltrate was present in these areas (not shown). Within the injury site (Fig. 4), there was a marked disruption of normal nerve architecture, with the severity of the abnormalities dependent on the proximity of the nerve fascicle to the suture material. Those nearest the ligature (Fig. 4A and B) demonstrated a characteristic increase in endoneurial tissue, and numerous thin myelinated fibers. In nerve fascicles adjacent to the injury site, but outside of the ligature (Fig. 4C), an increase in endoneurial tissue continued to be a prominent feature, and several of the larger myelinated fibers appeared to be undergoing degeneration. Numerous macrophages containing prominent phagosomes were also observed in these areas (Fig. 4D).

Cutaneous innervation

Immunocytochemical staining for PGP9.5, which is a general neuronal marker, revealed that chronic constriction of the IoN resulted in a partial deafferentation of both the upper (Fig. 5A and B) and deep (Fig. 5C and D) dermis of the ipsilateral

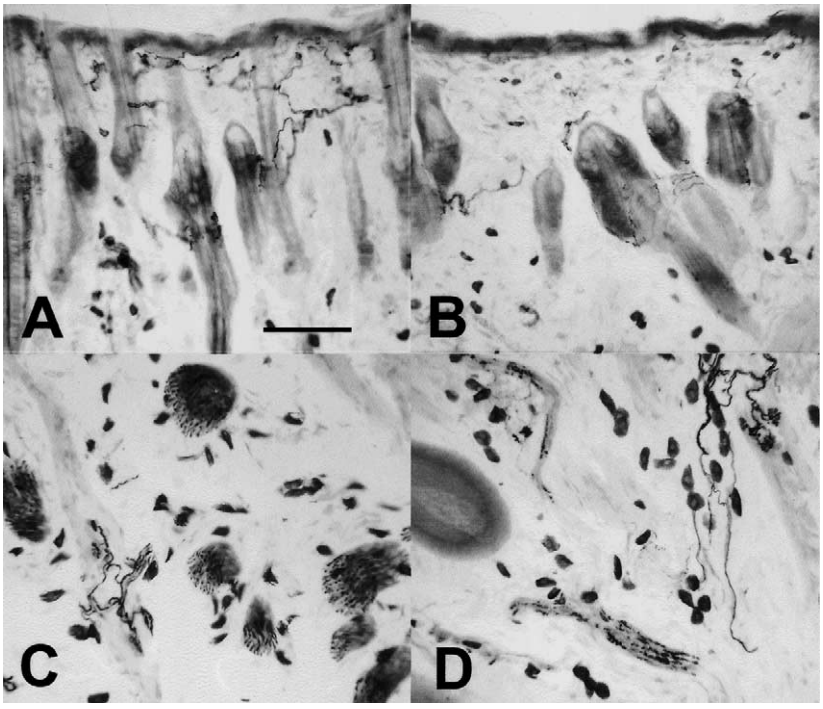

Figure 5 Representative light micrographs of skin from the affected IoN territory immunochemically stained for the presence of PGP9.5 immunoreactive nerve fibers 21 days after chronic constriction. (A) Upper dermis and epidermis from the contralateral, uninjured side. Numerous PGP9.5 immunoreactive fibers form a sub-epidermal plexus, and fibers extend into the epithelium. The bar represents 100 μm. (B) Upper dermis and epidermis from the ipsilateral, injury side. Note the marked decrease in the number of PGP9.5 immunoreactive fibers. Rare fibers can be seen extending into the epidermis. (C) Deep dermis from the contralateral, uninjured side. Large bundles of PGP9.5 immunoreactive fibers are present. Since many of these fibers are seen to be ending at motor end-plates on muscle fibers of the upper lip, they are assumed to be motor nerves. (D) Deep dermis from the ipsilateral, injured side. The large nerve fascicles are distinguished only by a sparse, granular PGP9.5 immunoreactivity. In contrast, motor fibers (including motor end-plates) appear intact.

vibrissae pad. The extent of deafferentation within the upper dermis was highly variable. Regions that were essentially devoid of PGP9.5 immunoreactive fibers were adjacent to areas with a normal appearing pattern of innervation. In the deep dermis, the large nerve bundles that normally enter the vibrissae were virtually absent on the injured side. Although a few large PGP9.5 immunoreactive nerves were present, PGP9.5 staining was irregular, which was suggestive of extensive Wallerian degeneration. Several large PGP9.5 immunoreactive nerve bundles in the deep dermis appeared to be unaffected by IoN constriction. Since these nerves were seen to be ending at motor end-plates on muscle fibers of the upper lip, they were assumed to be motor nerves.

Discussion

In the rat, constrictive injury to the IoN results in pain behaviors that resemble those seen in clinical pain conditions, such as secondary trigeminal neuralgia and post-herpetic neuralgia.[19–24] However, in most previous studies of experimental trigeminal nerve injury, the standard behavioral paradigm used to monitor the development of neuropathic pain has been to measure the response to mechanical stimulation. The results of the present study demonstrate that an enhanced response to a chemogenic challenge also develops after chronic constriction of the IoN. This orofacial inflammatory hypersensitivity occurs at a post-injury time when mechanical allodynia has been documented.[22] LaBuda et al.[5] recently reported similar enhanced responses to formalin in the L5 spinal nerve ligation model of neuropathic pain.

Curiously, IoN constriction did not significantly affect weight gain in this study, which is often thought to be a common, nonspecific behavioral sign of ongoing persistent pain. Vos et al.[19] reported that the average daily weight gain was lower in IoN compared with sham-injury rats, and that 130 days after IoN constriction mean body weight was significantly less in constriction injury rats than in sham-injury controls. However, the reduction in weight gain following IoN injury was less that 1 g per day. In our model of orofacial neuropathic pain, mechanical allodynia does not appear until 7–10 days after IoN constriction.[22] Thus, cumulative differences in weight gain that might have been seen over a longer time period were likely too small to be observed during the relatively short experimental period.

This points to an important limitation of this study, i.e. the selection of a single time point (21 days post-injury) for the behavioral observations. Neither the time of onset nor the duration of the inflammatory hypersensitivity (days after injury) are know at this time, and the clinical significance of our findings will largely reflect the extent to which neuropathic injuries either enhance, or even possibly diminish, the long-term response to inflammatory challenge.

Directed grooming has been a standard quantitative measure used in the formalin test for orofacial pain, and directed grooming in both the CCI and sham-injury groups during the early and late phases were similar to those previously reported for non-injured rats.[15,18] However, in CCI rats there was also a significant increase in a variety of behaviors that were seldom exhibited by sham-injury animals. These additional behaviors, which can be elicited in normal rats by simply increasing the formalin concentration (unpublished observations), included flinching (head and whole body), spontaneous vocalization, trismus, vigorous shaking followed by licking of the forepaws, and shielding of the face while tightly shutting both eyes. All of these behaviors suggest moderate to severe distress, and thus are potentially indicative of spontaneous, severe and possibly widespread pain and dysaesthesia.

Control experiments (saline injection and contralateral formalin injection) established two additional characteristics of the observed inflammatory hypersensitivity. First, physical damage per se (due to either needle penetration or injection volume) was not responsible for any of the observed nocifensive behaviors. Second, unlike findings for mechanical allodynia,[21,22] the enhanced response to formalin was a unilateral, rather than a bilateral, phenomenon.

As noted by LaBuda et al.,[5] the typical response to subcutaneous formalin injection can be divided into an early phase (activation of A_δ and C nociceptive fibers) and a late phase that results from the recruitment of ''silent'' nociceptors and non-injured afferents as part of the inflammatory process. The mechanisms responsible for the development of inflammatory hypersensitivity after chronic constriction injury are not known at this time, but they are undoubtedly related to the effects of nerve injury and inflammation that underlie the development and maintenance of neuropathic pain.[9] In particular, abnormal sensory inputs (e.g. the initial ''injury discharge'', spontaneous activity and mechanical sensitivity of the regenerating nerve fibers within the neuroma) contribute to the development of sensory disturbances and central sensitization.[25,26]

Central sensitization is critical to the development and maintenance of hyperalgesia and allodynia in a variety of persistent pain states, and it also plays a role in the response to orofacial noxious

stimulation. However, Dallel et al.[27] demonstrated that in the formalin test, both the first and second phases of the nociceptive response require input from primary afferents. Nonetheless, central sensitization, as a result of abnormal peripheral activity, was considered to be a contributing factor to the magnitude of the second phase.

In both the present study, and the recent work of LaBuda et al.,[5] the behavioral responses were monitored for only 45 min, which leaves some questions unanswered. The inflammatory response surrounding the area of formalin injection persists for a significantly longer time. Swelling of the upper lip, for example, could still be seen on the following day. Thus, it will be important to monitor behavioral responses (e.g. food intake and the response to von Frey stimulation) and determine the duration and severity of the hyperalgesia (both primary and secondary) that is evoked by the chemogenic challenge. In addition, little is known about the effect of deafferentation on the inflammatory response per se.

Following nerve injury, the distal portion of the affected axons undergo Wallerian degeneration, and while some axons do indeed regenerate and reinnervate their original territory, there is often a partial deafferentation of the peripheral sensory field. Clinically, partial deafferentation is often associated with chronic pain syndromes, e.g. postherpetic neuralgia, but the mechanisms underlying deafferentation pain are not clearly understood. It has been hypothesized that deafferentation results in anatomical, neurochemical and physiological changes in the dorsal horn (substantia gelatinosa) and the brainstem (trigeminal nucleus caudalis) that affect pain processing.[9,28]

Partial deafferentation the IoN territory was suggested by the apparent reduction in the number of PGP9.5 immunoreactive fibers present in the ipsilateral cutaneous tissues. Large PGP9.5 immunoreactive fibers, which were presumed to be motor fibers due to their being seen ending at motor end-plates, were unaffected. This was expected, because the IoN is a largely sensory nerve that carries no motor and only a few autonomic fibers. At the injury site itself, only thin, lightly myelinated fibers were observed, which is a characteristic feature of regenerating nerves within a neuroma.[29,30] Robinson and colleagues[31,32] demonstrated that constriction of the inferior alveolar or lingual nerve, which are largely sensory in nature, results in the development of both spontaneous activity and mechanical sensitivity originating from myelinated fibers at the injury site.

It must be emphasized, however, that our morphological observations are only qualitative in nature, and the actual extent of deafferentation was not determined. Additional, carefully controlled morphometric studies will be required to determine the precise relationship between specific types of fiber loss (A_β, A_δ or C) and the development and severity of pain behaviors.

In addition to overt structural damage, there was an inflammatory response at the injury site that had not completely resolved by 21 days. Numerous macrophages and mast cells were observed both proximal and distal to the ligature, and there was a marked inflammatory infiltrate surrounding the remaining fragments of chromic gut (not shown). Pro-inflammatory cytokines, prostaglandins and NGF synthesized by macrophages, Schwann cells and other immune cells sensitize nociceptive nerve fibers,[33] which contributes to hyperalgesia in inflammatory models of pain.[34] Inflammation also plays a role in the development of neuropathic pain. For example, in the sciatic nerve, focal inflammation in the absence of structural damage results in abnormal pain sensitivity in the hind paw.[35] Additionally, increased pro-inflammatory cytokine gene expression is associated with hyperalgesia and mechanical allodynia[36] and endoneurial injection of tumor necrosis factor-α evokes neuropathic pain behaviors.[37] Finally, Anderson and Rao[22] demonstrated an increase in both IL-6 and NGF following constriction of the IoN.

Irrespective of the mechanisms involved, the present data provide additional evidence that enhanced inflammatory pain may be one of the consequences of neuropathic injury. The implications of these findings with respect to the clinical management of orofacial pain are likely to be significant, and studies involving shorter and longer time points after IoN injury, and other inflammatory pain models that more closely resemble clinical inflammation are warranted.

Acknowledgements

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