PAIN

Pain 86 (2000) 163–175

www.elsevier.nl/locate/pain

Alleviation of mechanical and thermal allodynia by $CGRP_{8-37}$ in a rodent model of chronic central pain

Adrianne D. Bennett, Kathy M. Chastain, Claire E. Hulsebosch*

Marine Biomedical Institute, Anatomy and Neurosciences, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-1069, USA

Received 3 February 1999; received in revised form 17 November 1999; accepted 13 January 2000

Abstract

$CGRP_{8-37}$ is a truncated version of calcitonin gene-related peptide (CGRP) that binds to the CGRP receptor with similar affinity but does not activate the receptor and is a highly selective CGRP receptor antagonist. CGRP and activation of its receptor appear to play a role in peripheral inflammatory and neuropathic models of pain although there is considerable controversy. The aim of this study was to examine possible anti-nociceptive effects of $CGRP_{8-37}$ on a model of chronic central neuropathic pain known to develop weeks after spinal hemisection. Adult male Sprague–Dawley rats were given a spinal hemisection ($N = 34$) or a sham surgery ($N = 10$) at the T13 spinal segment. An externally accessible PE-10 intrathecal catheter that terminated at T13 was used for drug delivery. Animals were allowed to recover for 4 weeks at which time the hemisected animals displayed mechanical and thermal allodynia bilaterally, in both forelimbs and hindlimbs. $CGRP_{8-37}$ was delivered just prior to a testing session in 1, 5, 10, or 50 nM doses in artificial cerebral spinal fluid in 10 μl volumes. $CGRP_{8-37}$ was effective in alleviating mechanical and thermal allodynia in a dose-dependent manner ($P < 0.05$). The 50 nM dose was most efficacious for both forelimb and hindlimb responses ($P < 0.05$). The period of efficacy was 10 min to onset for a duration of 20 min. Post-drug washout responses were not statistically significant compared to pre-drug responses. The sham control groups demonstrated no statistically significant difference at any dose of $CGRP_{8-37}$ when compared to pre-surgical baseline values. In conclusion, $CGRP_{8-37}$ is effective in abolishing mechanical and thermal allodynia produced by spinal hemisection. Consequently, the CGRP receptor may play a role in chronic central neuropathic pain and offers a novel therapeutic approach to managing chronic central pain.

Keywords: Spinal cord injury; Dorsal root ganglia; Allodynia; Rat; Central pain

1. Introduction

CGRP is a peptide 37 amino acids long that can be divided into two types, i.e. α-CGRP and β-CGRP (Mulderry et al., 1988), where α-CGRP is the product of tissue-specific alternative RNA splicing of the calcitonin gene (Rosenfeld et al., 1983) and β-CGRP is encoded by a separate gene (Amara et al., 1982, 1985). With respect to the primary afferent sensory system, α-CGRP is located in peripheral projections (Ishida-Yamamoto and Tohyama, 1989; Hökfelt et al., 1992), in the somata of small diameter dorsal root ganglion neurons (Cameron et al., 1988; Hökfelt et al., 1992), and in central projections in laminae I, II outer (IIo), and V of the dorsal horn (Gibson et al., 1984; McNeill et al., 1988; Ishida-Yamamoto and Tohyama, 1989; Hökfelt et al., 1992). CGRP is also located in the intermediolateral and ventral horns of the spinal cord in sympathetic and motor neurons, respectively (Marti et al., 1987; Senba and Tohyama, 1988). Selective surgeries have established that CGRP in the dorsal horn is of primary afferent in origin in normal and chronically deafferented mammalian spinal cords (Chung et al., 1988; McNeill et al., 1991). Both immunocytochemical and in situ hybridization techniques confirm that CGRP is produced in small diameter neurons in the dorsal root ganglion (DRG) which give rise to unmyelinated (C) and thinly myelinated (Aδ) fibers (Willis and Coggeshall, 1991). This subpopulation of primary afferent fibers has been implicated in the transmission of pain, temperature sensation, and other modalities including noxious and non-noxious mechanical stimuli (Willis and Coggeshall, 1991).

The observation that CGRP is involved in nociceptive behavior is supported by several experiments. For example, intrathecal injections of CGRP produce significant reductions in the latency of hindpaw withdrawal in response to noxious thermal and mechanical stimulation (Oku et al., 1987; Crid-

* Corresponding author. Tel.: +1-409-772-2939; fax: +1-409-762-9382.

E-mail address: cehulseb@utmb.edu (C.E. Hulsebosch)

0304-3959/00/$20.00
PII: S0304-3959(00)00242-6

land and Henry, 1988, 1989). In other examples, CGRP delivered to the spinal cord results in thermal hyperalgesia and other nociceptive behavior in rats (Oku et al., 1987; Christensen et al., 1996a,b). Our laboratory found that intrathecal injection of 10 μl of 100 nmol of CGRP into naive adult rats produced writhing, spontaneous biting and vocalization, evidence that CGRP alone can produce nociceptive behaviors. By contrast, intrathecal administration of antiserum developed against CGRP normalizes the responses to thermal and mechanical noxious stimuli in rats rendered experimentally hyperalgesic by either adjuvant or carrageenan injections into the paw (Kuraishi et al., 1988; Kawamura et al., 1989; Satoh et al., 1992). In addition, intrathecal administration of the CGRP antagonist, $CGRP_{8-37}$, into normal rats inhibits both thermal and mechanical nociception (Yu et al., 1994). More specifically, these data suggest that intraspinal CGRP is directly involved in mediating nociceptive behavior of either central or peripheral origin.

Previous work from our lab using a rodent model of chronic central pain produced by spinal hemisection demonstrated an increase in CGRP immunoreactivity in the dorsal horn of several thoracic segments 2 weeks after surgery, which persisted for at least 108 days post-injury (Christensen and Hulsebosch, 1997a,b). Work by others demonstrated an increase in CGRP immunoreactivity in the dorsal horn of thoracic and lumbar segments (T1 through L6) after spinal thoracic transection (Krenz and Weaver, 1998; Krenz et al., 1999). However, there is no direct evidence in the literature that indicates the involvement of CGRP in chronic central pain behavior. The present study tests whether blockade of the CGRP receptor, by a selective antagonist, reduces the abnormal nociceptive behavior observed in this rodent model of chronic central pain produced by spinal hemisection (Christensen et al., 1996a; Christensen and Hulsebosch, 1997a). We used the antagonist $CGRP_{8-37}$, which is a truncated version of CGRP that binds to the CGRP receptor with approximately the same affinity as CGRP, but does not affect the receptor signal transduction sequence (Chiba et al., 1989; Van Rossum et al., 1994). Previous behavioral studies demonstrated that spinal hemisection produces mechanical and thermal allodynia in both forelimbs and hindlimbs that develops over several weeks, is stable by 28 days and persists for up to 108 days (Christensen et al., 1996a; Christensen and Hulsebosch, 1997a). In the present set of experiments, we administered $CGRP_{8-37}$ in rats spinally hemisected and after the development of behavior consistent with chronic central pain, to test for attenuation of the mechanical and thermal allodynia behaviors.

2. Materials and methods

2.1. Surgical procedures

Male Sprague–Dawley rats (175–200 g) were obtained from Harlan Sprague-Dawley, Inc. and housed with a reversed light/dark cycle of 16 h/8 h where the dark cycle began at 07:00 h. Since the rats are nocturnal animals, the behavioral tests occurred during the dark cycle or their 'awake' period in the circadian rhythm. The rats were acclimated and given behavioral tests to determine baseline behavior as described later, and then divided into two groups, i.e. sham controls ($N = 10$) and spinal hemisected rats ($N = 7$–10 per dose; $N = 7$ each for the 1 and 5 nM doses, and $N = 10$ each for the 10 and 50 nM doses for a total of 34 rats). Rats were deeply anesthetized by intraperitoneal injection of ketamine and xylazine (75 and 15 mg/kg, respectively) as determined by the absence of the tail and paw withdrawal reflex to pinch and the absence of corneal blink reflex. The spinal cord was hemisected at T13 on the left side by the following procedure: following palpation of the dorsal surface to locate the cranial borders of the sacrum and the dorsal spinous processes of the lower thoracic and lumbar vertebrae, the T11, T12 and T13 laminae were determined by locating the last rib, which attaches to the cranial end of the T13 vertebrae. The surgical field was shaved and prepared with betadine, and a longitudinal incision was made exposing several segments. A laminectomy was performed at vertebral level T11, the lumbar spinal cord was identified with accompanying dorsal vessel, and the spinal cord was hemisected at T13, cranial to the L1 dorsal root entry zone with a #11 scalpel blade without damage to the major dorsal vessel or vascular branches. Iridectomy scissors were used to ensure the completeness of the hemisection. For the sham group, similar procedures were performed with the exception that the spinal cord was not hemisected.

At the time of surgery, a sterilized, artificial cerebrospinal fluid (ACSF; pH 7.4) filled PE-10 intrathecal catheter was threaded through the intrathecal space, after partial laminectomy through a dural slit, three vertebral segments rostral (T9) to the hemisection site. The end of the catheter terminated at the hemisection site. Visual confirmation of the end of the tube assured correct placement. A loose knot was placed in the tubing cranial to the dural entry zone, fixed by application of cyanoacrylic glue, and anchored in place with two polypropylene sutures (5-0) to the erector spinae musculature. The musculature and the fascia were then sutured and the skin was apposed by autoclips. An 8 cm length of tubing leader was left exposed through the skin of the surgical site and the end was heat sealed closed with heated forceps. Intrathecal delivery tubes were inserted in both sham and hemisected groups. The rats were eating and drinking within 3 h after surgery. Animals had to be housed individually after surgery to prevent catheter removal by cage mates. To ensure the general health of the rats, the animals were weighed daily. Weight loss was minimal, occurred acutely over the first 2 postoperative days and was not greater than 5% of the total body weight. By the third postoperative day, the animals demonstrated normal weight gains. The protocols used were approved by the

University of Texas Medical Branch Animal Care and Use Committee (UTMB-ACUC).

2.2. Behavioral procedures

Locomotor function was observed and recorded using the modified open field test first developed by Tarlov and Klinger (1954) and recently modified into the Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale (Basso et al., 1995) to ensure that motor recovery occurs and does not impair the somatosensory behavioral tests. The score is based on locomotor ability following experimental SCI in rodent models. Briefly, the BBB scale is a 21 point scale which ranges from 0, which is no observable hindlimb movement, to 21, which is consistent and coordinated gait, hindlimb parallel paw with consistent trunk stability. Scores from 0 to 7 rank the early phase of recovery with the return of isolated joint movements of the three joints (hip, knee, ankle), scores from 8 to 13 describe the intermediate recovery phase with the return of paw placement, stepping and forelimb–hindlimb coordination, and scores from 14 to 21 rank the late phase of recovery with the return of toe clearance during the step phase, predominant paw position, trunk stability and tail position. Thus, the BBB score for spinally injured rats allows assessment of hindlimb recovery. In addition, any decrease in the BBB score in response to a drug test would suggest sedation. On the day after surgery, all animals scored 0 or 1 for the hindlimb ipsilateral to the hemisection. Animals with higher scores, or animals with contralateral hindlimb impairment were excluded from the study. Left and right hindlimbs were assessed separately. Forelimbs demonstrated no changes in locomotion due to surgery or in antagonist/vehicle experiments. The recovery of the hindlimb ipsilateral to the hemisection allows a temporal measure of the status of functional recovery. The somatosensory tests begin once the locomotor recovery period has reached maximum recovery and has stabilized as determined by the BBB scores, considered to be an indicator of motor recovery after spinal cord injury (Basso et al., 1995).

Behavioral tests representing mechanical and thermal allodynia were performed preoperatively and postoperatively for both forelimbs and hindlimbs. Sham animals were tested for allodynia-like behavior as a control for the criteria used by the behavioralist and to control for the possibility that a learned response from a repeated stimuli could produce an increased number of paw withdrawals unrelated to the development of allodynia. Prior to the onset of behavioral testing, all animals were environmentally acclimated to the clear Plexiglas cubicle testing apparatus (8 × 8 × 18 cm) for 4 h daily for 3 days. The preoperative testing began 3 days prior to surgery and was used to establish both individual and group baseline behaviors. The data from each limb were collected independently. Because there was no statistically significant difference between left and right forelimbs, the data were combined. Only data from the contralateral hindlimbs were analyzed. The tests were performed postoperatively once a week for 4 weeks prior to the drug tests. The development of mechanical and thermal allodynia after hemisection is found to be well established bilaterally in forelimbs, hindlimbs and on the dorsal trunk by 4 weeks postoperatively (Christensen et al., 1996a; Christensen and Hulsebosch, 1997a). Mechanical allodynia of the glabrous skin of the paw was quantified by measuring the number of brisk paw withdrawals in response to normally innocuous mechanical stimuli (Choi et al., 1994). The subthreshold mechanical stimuli were von Frey filaments with bending forces of 4.78 and 9.96 mN. In addition, suprathreshold mechanical stimuli, a von Frey filament with a bending force of 49.9 mN, and a mechanical noxious stimulus, a pin, were used. In human perceptual terms, these mechanical forces represent a subthreshold stimulus, a light touch, a poke, and a noxious prick, respectively. The pin did not cause overt tissue damage and was selected to control for sedation effects and unwanted drug-induced changes in behavior in response to stimuli that were noxious.

To perform these tests, rats were placed inside the Plexiglas boxes on an elevated, fine metal screen and acclimated for 60 min prior to testing. The von Frey filament was applied from underneath the metal mesh floor, through the mesh, to the plantar surface of the glabrous skin of the paw for each limb. A single trial consisted of 10 applications of von Frey filament, applied once every 3–4 s. A response is defined as a withdrawal of the stimulated paw with accompanying attention of the rat to the stimulus mediated by supraspinal pathways such as head turning, disengaging behavior from ongoing activity, biting the von Frey hair, etc. The mean occurrence of paw withdrawal in each of the trials was taken for each limb in each rat as a repeated measure and was expressed as a mean number of responses where 0 indicated no paw withdrawal and 10 indicated the maximum number of paw withdrawals. The occurrence of paw withdrawal of the 10 trials was recorded and this value was then normalized to the pre-surgical baseline at the level of the individual animal to derive the change in response to the surgery and the surgery with drug treatment (number of paw withdrawals – baseline). The changes were analyzed and graphed as group data for between group comparisons where the pre-surgical baseline is zero.

Thermal allodynia was measured by the latency of paw withdrawal to noxious stimuli as previously described by Bennett and Xie (1988) and Hargreaves et al. (1988). Animals were placed in a Plexiglas box on an elevated glass plate under which a light box is placed. A radiant heat stimulus was applied by concentrating a beam of light through a hole in the light box onto the plantar surface of the paw of each limb through the glass plate. The light beam is turned off automatically by a photocell when the animal lifts the limb, allowing the measurement of time between the start of the light beam and the paw withdrawal. A response is defined as a paw withdrawal with head turning

and paw licking. The time is defined as the paw withdrawal latency. Five minutes were allowed between each trial and three trials were averaged for each limb. The results for the thermal tests were then normalized to the pre-surgical baseline at the level of each individual animal to determine the change in paw withdrawal latency in response to the surgery and the surgery with drug treatment. These data were analyzed and graphed as group data where pre-surgical baseline is displayed as 0, and the data were compared as change relative to the pre-surgical baseline responses for between group comparisons.

2.3. Drug testing

Both drug and vehicle injections were administered blind and the injection schedule was randomized as to the order given and with regard to drug or vehicle. Dilutions of 1, 5, 10, and 50 nM were made for $CGRP_{8-37}$ in ACSF (pH 7.4). A test dose of 10 μl of one concentration was injected into the intrathecal catheter followed by a wash of 10 μl ACSF and the behavior was immediately assessed. Drug challenges began after 28 days post-surgery, at which time the responses to the mechanical and thermal stimuli were maximal and stable, as determined empirically (Christensen et al., 1996a). Prior to the tests of the various drug concentrations, the onset and duration of drug effect were empirically determined to be 10 min for onset and 20 min for duration of the drug effect for the doses of $CGRP_{8-37}$ used in this study. Consequently, each behavioral test was done within the 20 min window of drug efficacy. At all tested doses, the post-drug responses always returned to pre-drug responses, even for the high doses (see Section 3); thus, there was no evidence for long-term desensitization. Some animals received more than one test injection, to test for possible desensitization, sensitization or morbidity with repeated doses (data not shown). The results were not statistically significant from the results for each dose and for drug washout responses. For these animals, at least 24 h were allowed between each drug challenge to allow sufficient time for physiological washout of the drug. Behavioral testing was done blind to the surgical status or the vehicle or dose injection being tested. The sham data control for a learned response from repeated peripheral stimuli that might result in an increased number of paw withdrawals unrelated to the development of allodynia and for behavioral changes that might occur due to injection stress of either the vehicle or the vehicle containing $CGRP_{8-37}$. The effects of stress might be either hypersensitivity or analgesia. Vehicle alone injections were given as a control to both groups and the responses were not statistically significant when compared to uninjected vehicle behavioral data for either sham control or spinal hemisected groups. Post-drug testing was performed and behavioral values were not statistically different when compared to 30 day post-surgical, pre-injection behavioral values for both sham control and spinal hemisected groups. Finally, as a control for the specificity of $CGRP_{8-37}$ in the observed response, a group of hemisected animals ($N = 5$) was tested after mechanical and thermal allodynia was established with a combination of 5 μl of 100 nM $CGRP_{8-37}$ and 5 μl of 1000 nM CGRP. In these animals, the behavioral data in the mechanical behavioral tests for all von Frey strengths and the pin demonstrated no statistically significant difference between behavioral data collected pre-injection, during the 20 min period of drug efficacy, and post-injection for either forelimbs or hindlimbs. For the thermal behavior tests, there were similarly no statistically significant differences.

CGRP was administered by intrathecal catheter in naive rats as a control to directly demonstrate a nociceptive role for CGRP. A dose of 100 nmol in 10 μl buffered ACSF resulted in spontaneous vocalization and writhing accompanied by biting and scratching at the dermatomes corresponding to the termination site of the catheter. This behavior continued for 7 min. A mechanical stimulus (von Frey filament of 4.78 mN) was applied to the trunk and the number of vocalizations (out of 10 stimuli) increased from 0 (pre-injection) to 10 by 15 min post-CGRP injection (data not shown). Additionally, the latency of paw withdrawal to the radiant heat stimulus decreased from an average of 22 s (pre-injection) to 12 s. Only two animals were tested as the UTMB-ACUC prohibits experiments that are 'painful or distressful'; thus, these experiments were terminated for humane reasons. Since $CGRP_{8-37}$ is a potent vasoconstrictor (Jansen-Olesen et al., 1996; Perren et al., 1996; Kagstrom and Holmgren, 1998) and intrathecal injections of $CGRP_{8-37}$ produce a rapid decrease in body temperature in normal mice (Saxen et al., 1994) which, if this occurs in rats, may confound the somatosensory tests in the present experiments, we tested for possible changes of temperature on the glabrous skin of the fore and hindpaws and additionally, monitored rectal temperatures. We found no evidence of a statistically significant difference in temperatures pre-drug compared to during the therapeutic window of drug efficacy in shams or hemisected animals even at the 50 nM dose (data for the hemisected 50 nM group (mean ± SE): forepaws, pre-drug 31.6 ± 0.9°C, drug effect 30.5 ± 0.8°C; hindpaws, pre-drug 30.9 ± 0.3°C, drug effect 30.8 ± 0.3°C; rectal temperature, pre-drug 38.8 ± 0.4°C, drug effect 39.2 ± 0.2°C).

2.4. Statistical analysis

All statistical tests were evaluated at the alpha level of significance of 0.05. The data from these procedures were tested for statistical significance using more conservative non-parametric tests, thus avoiding issues of normality and equal variance of the data distribution. We used Kruskal–Wallis ANOVA on ranks followed by a pairwise comparison using Dunn's method since the numbers of individuals per group were different. Data management and statistical analyses were performed using SigmaStat™ software. Correlations of behavioral outcomes with doses were

tested by either Spearman rank order correlation or Pearson product moment correlation tests. All values are graphed as means (X) ± standard errors (SE).

3. Results

3.1. Locomotor scores

All animals started with hindlimb BBB scores of 21 (Fig. 1). Spinal cord hemisection resulted in ipsilateral hindlimb paralysis. On the day after surgery, the hemisected group scored 0.7 ± 0.3 on the ipsilateral hindlimb. By post-surgical day 7, animals recovered considerable motor function (17.4 ± 5.3 out of 21). The BBB scores decreased slightly at post-surgical day 14 from 17.4 ± 5.3 to 15.2 ± 0.4, which was not statistically significant, and remained at similar values for the remainder of the study. In order to determine whether $CGRP_{8-37}$ had an adverse effect on motor function, BBB scores were assessed at every drug injection concentration. There was no statistically significant difference in BBB scores at any dose, including the 50 nM dose at which maximum analgesia was observed.

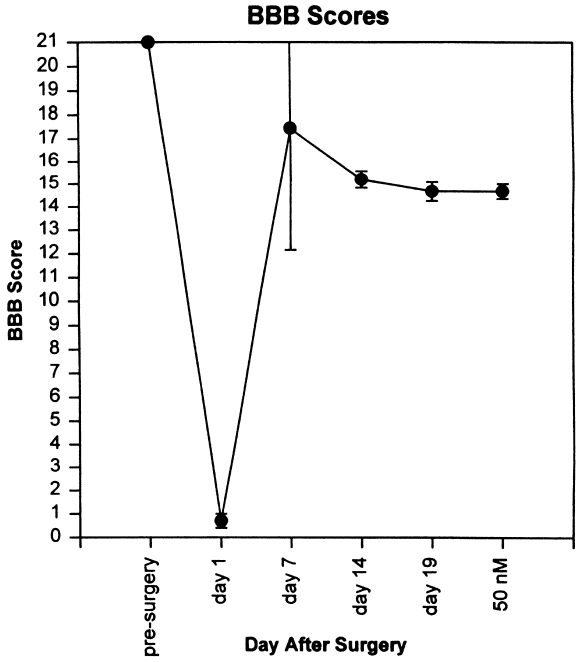

Fig. 1. Motor scores for the ipsilateral hindlimb of the hemisection animals displayed as the mean ± SE. Twenty-one represents normal locomotion and 0 represents no observable movement. On post-surgical day 1, the animals scored a 0.7 ± 0.3. By day 7 motor scores had increased significantly, and by day 19 evened-out to remain significantly improved for the remainder of the study. Note that even the high dose of 50 nM $CGRP_{8-37}$ did not produce a measurable change in limb function which suggests no sedative effect from this dose.

3.2. Intrathecal catheter

It has been suggested that the presence of intrathecal catheters induces a progressive development of gliosis, fibrosis and scarring in the vicinity of the terminus of the indwelling catheter in rats and patients (Mercadante, 1999). We have not seen evidence of fibrosis at the terminus of the indwelling catheter in rats that we have autopsied although histological sections do demonstrate a thin layer of fibrosis surrounding the tube but not encroaching on the cord. The indwelling catheter does not appear to affect the spinal hemisection response to injury (fibrosis, tethering, etc.) and does not change the behavioral outcomes as compared to responses for groups of spinal hemisectioned rats without indwelling catheters. Additionally, no blockage of the tube was observed that could have resulted from fibrosis at the catheter opening. The only blockage occurred at the exposed end due to crystallization from evaporation; otherwise all injections flowed easily through the tube.

3.3. Mechanical sensitivity

Spinal cord hemisection produced a three-fold increase to 3.4 ± 0.2 and 2.8 ± 0.2 (compared to a baseline of 0) in the forelimb paw withdrawals to the low intensity von Frey filaments, 4.78 and 9.96 mN (Fig. 2A,B), respectively, and this increase is statistically significant ($P < 0.05$). All four concentrations of $CGRP_{8-37}$ created statistically significant decreases in the paw withdrawals when compared to post-surgical values ($P < 0.05$), with the most effective concentration being 50 nM, which brought the forelimb paw withdrawals back to responses that were not significantly different compared to pre-surgical baseline values. The post-drug test time point shows the paw withdrawals at 2.6 ± 0.4 and 3.8 ± 0.7 for each stimulus strength, which are not significantly different from the post-surgical time points recorded prior to the drug tests. The decreased responses were related to the increased doses in a dose-dependent manner as determined by Spearman rank order correlation analyses ($P < 0.05$). By contrast, the control responses for these same doses show that the $CGRP_{8-37}$ did not significantly affect the paw withdrawals in the sham control rats.

The responses to the 49.9 mN von Frey were somewhat different in that the number of forelimb paw withdrawals at the post-surgical time point were two-fold higher (1.7 ± 0.3) than baseline values and this increase is statistically significant ($P < 0.05$, Fig. 2C). The 1 or 5 nM dose of CGRP did not statistically alter the post-surgical response, whereas both the 10 and 50 nM concentrations decreased the number of paw withdrawals to values that were not statistically significant compared to pre-surgical values, i.e. −1.41 ± 1.2 and −1.28 ± 1.4, respectively. The drug treatment did not significantly change the paw withdrawal values for the control group. For the pinprick test, responses were maximal at baseline (10 out of 10), and hemisection did not alter the response to this stimulus. Treatment with $CGRP_{8-37}$ resulted in no statistically significant differences

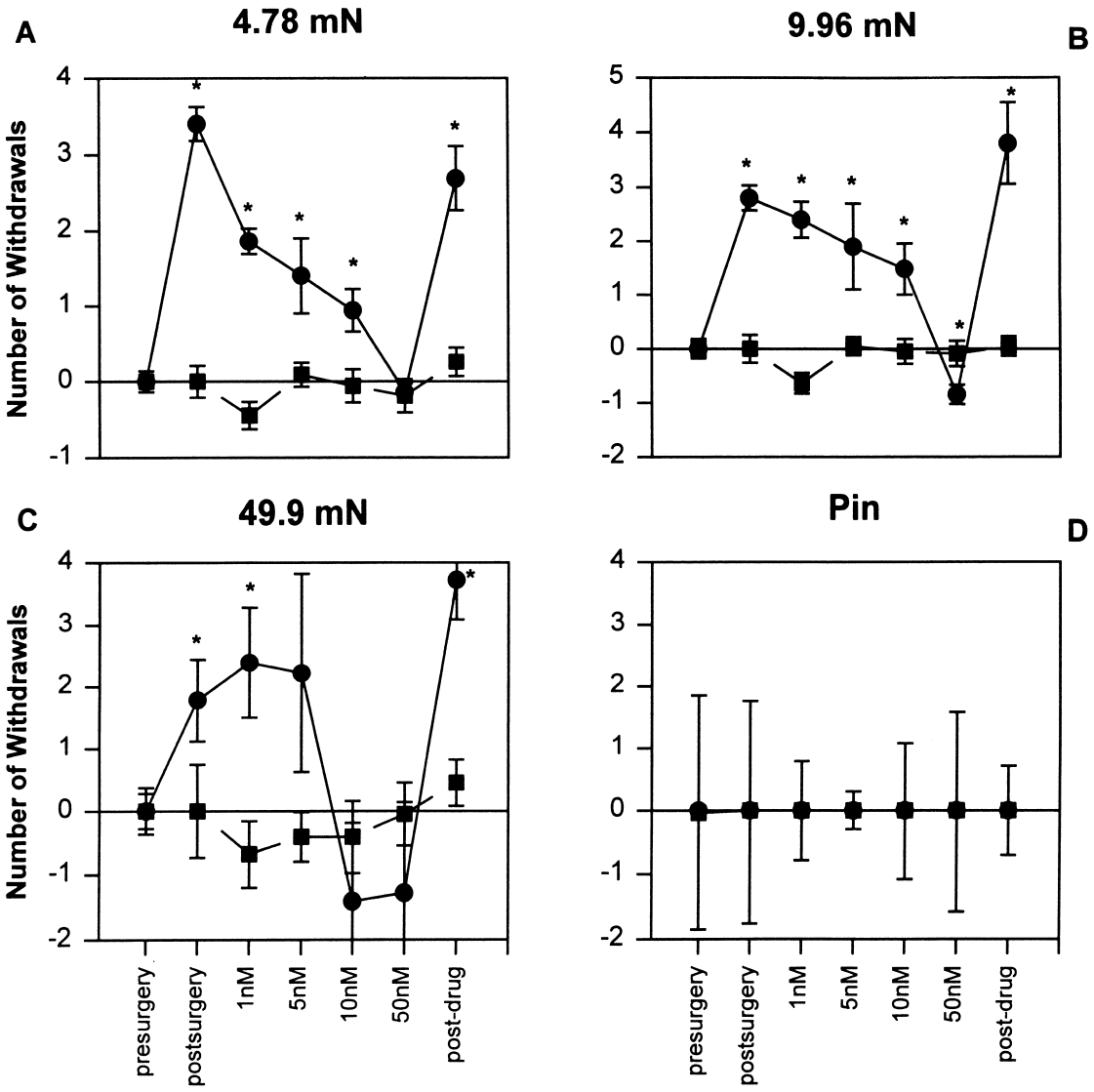

Fig. 2. Forelimb responses to various mechanical stimuli for the hemisected group (filled circles) and the sham control group (filled squares) displayed as the mean ± SE. Each of the von Frey stimulus strengths that were tested are displayed by the milli Newtons (mN) bending force generated. Note that the response to the tested doses of $CGRP_{8-37}$ were dose-dependent ($P < 0.05$) for the 4.78 mN (A), the 9.96 mN (B), but not for the 49.9 mN or the pin stimuli. The 50 nM dose reduced the number of paw withdrawals to values that were not statistically different from baseline values for both the 4.78 mN (A) and 9.96 mN (B) stimuli. The 10 and 50 nM doses were effective in reducing paw withdrawals for the 49.9 mN (C) stimulus. The pinprick stimulus (D) was maximally aversive and drug delivery did not produce any behavioral alterations, thus suggesting preservation of normally noxious responses. The post-drug recovery values demonstrated the reversible nature of the response and the specificity of the change in behavior to $CGRP_{8-37}$. The sham control groups did not have statistically significant changes compared to pre-surgical values for any dose tested. The asterisk (*) denotes statistical significance ($P < 0.05$) compared to pre-surgical control values.

in pinprick response at any dose for either the spinal hemisected or the sham control groups (Fig. 2D).

Fig. 3 graphs the change in paw withdrawals of the hindlimbs for the hemisected group compared to the hindlimbs for the control group. The post-surgical paw withdrawals increased three- and four-fold (2.7 ± 0.2 and 4.5 ± 0.43) for the 4.78 and 9.96 mN von Frey filaments, respectively (Fig. 3A,B). The 1 nM dose of $CGRP_{8-37}$ decreased the paw withdrawals to 1.6 ± 0.0 and 2.3 ± 0.3, respectively for each stimulus and this is statistically significantly different from the post-surgical time point ($P < 0.05$); however, these responses are significantly above the baseline and control values. The 5 nM dose decreased the paw withdrawals further to 1.2 ± 0.5 and 1.87 ± 0.9. However, unlike the forelimb responses, both the 10 and 50 nM doses decreased the paw withdrawal responses to values that were not significantly different from baseline or control values. That the paw withdrawals were dose-dependent in the hemisected group is supported by Spearman rank order correlation statistics ($P < 0.05$). No significant changes occurred in the sham control group at any dose tested.

The pattern of drug interaction for the high intensity 49.9

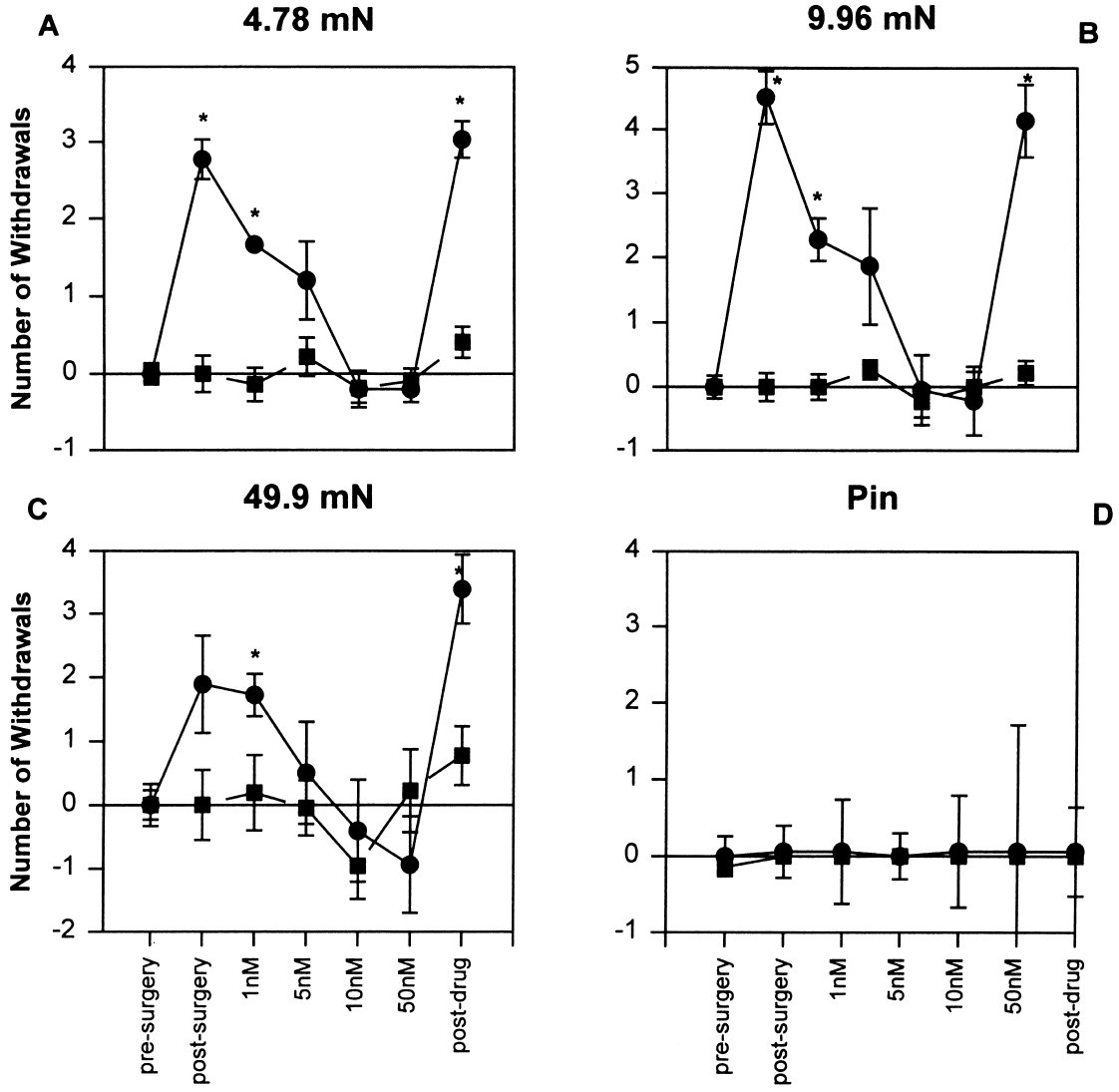

Fig. 3. Hindlimb responses to mechanical stimuli for the hemisected group (filled circles) and the sham control group (filled squares) displayed as the mean ± SE. Each of the von Frey stimulus strengths that were tested are displayed by the milli Newtons (mN) bending force generated. Note that the response to the tested doses of $CGRP_{8-37}$ were dose-related. Both the 10 and 50 nM doses were effective at reducing paw withdrawals in the 4.78 mN (A) and 9.96 mN (B) tests. In the 49.9 mN (C) test these same doses reduced paw withdrawals to below baseline responses. For the 4.78, 9.96 and 49.9 mN von Frey strengths, there was a statistically significant dose-dependent response ($P < 0.05$) to the different doses tested. The drug delivery did not produce any alteration in response to the pinprick (D) test. The post-drug recovery values demonstrated the reversible nature of the response and the specificity of the change in behavior to $CGRP_{8-37}$. The sham control groups did not have statistically significant changes compared to pre-surgical values for any dose tested. The asterisk (*) denotes statistical significance ($P < 0.05$) compared to pre-surgical control values.

mN von Frey filament was very similar to that seen for the low intensity filaments (Fig. 3C). The 5, 10 and 50 nM doses reduced the number of paw withdrawals to a point not significantly different from pre-surgical or control responses, to 0.5 ± 0.8, -0.41 ± 0.8 and -0.94 ± 0.7, respectively. This implies that for the hindlimbs, the 5, 10 and 50 nM concentrations of $CGRP_{8-37}$ were sufficient to reduce the mechanical allodynia to the baseline, pre-surgical levels. The responses for the hemisected group, but not the sham control group, were dose-dependent as determined by Spearman rank order correlation statistics ($P < 0.05$). As in the forelimbs, treatment with $CGRP_{8-37}$ resulted in no statistically significant differences in pinprick response at any dose for either the spinal hemisected or the sham control groups. Thus, the tested doses of $CGRP_{8-37}$ did not significantly affect the paw withdrawal response to this blatantly noxious stimulus (Fig. 3D). With regard to the concern that repeated intrathecal injections affect subsequent responses, we have no evidence that this occurs since all responses beyond the temporal window of drug efficacy returned to values that were not significantly different when compared to post-surgical allodynic conditions. In addition, hemisected animals that were exposed to multiple doses of $CGRP_{8-37}$ showed a similar decrease in response and post-drug washout response as the animals in the treatment group.

As a control for the specificity of $CGRP_{8-37}$ in the observed response, a group of hemisected animals ($N = 5$) was tested after mechanical and thermal allodynia was established with a combination of 5 μl of 100 nM $CGRP_{8-37}$ and 5 μl of 1000 nM CGRP. In these animals, the behavioral data in the mechanical behavioral tests for all von Frey strengths and the pin demonstrated no statistically significant difference between behavioral data collected pre-injection, during the 20 min period of drug efficacy, and post-injection for either forelimbs or hindlimbs. The changes in responses compared to pre-surgical values for the forelimb withdrawal response during the period of $CGRP_{8-37}$/CGRP effect were 2.8 ± 0.47, 2.6 ± 0.38, 3.4 ± 0.53 and 0.0 for the 4.78, 9.96, 49.9 mN and pin, respectively. For the hindlimb responses, the change in withdrawal responses during the period of $CGRP_{8-37}$/CGRP effect were 3.3 ± 0.38, 4.7 ± 0.4, 2.4 ± 0.8 and 0.0 for the 4.78, 9.96, 49.9 mN and pin, respectively.

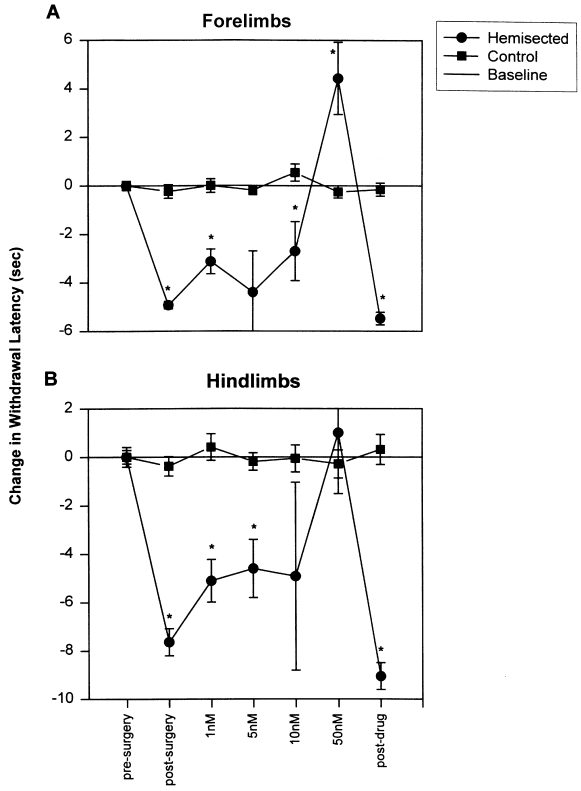

Fig. 4. Forelimb (A) and hindlimb (B) responses to radiant heat stimuli for the hemisected group (filled circles) and the sham control group (filled squares) displayed as the mean ± SE. The response of the hemisected groups to the tested doses of $CGRP_{8-37}$ were dose-dependent ($P < 0.05$). The 50 nM dose increased the withdrawal latency to above baseline levels in the forelimbs indicating hypoalgesia. The same dose increased the withdrawal latency to baseline levels for the hindlimbs. The post-drug recovery values demonstrated the reversible nature of the response and the specificity of the change in behavior to $CGRP_{8-37}$. The sham control groups did not have statistically significant changes compared to pre-surgical values for any dose tested. The asterisk (*) denotes statistical significance ($P < 0.05$) compared to pre-surgical control values.

3.4. Radiant heat sensitivity

Spinal hemisection resulted in post-surgical paw withdrawal latencies that were significantly decreased when compared to pre-surgical values for both forelimbs and hindlimbs ($P < 0.05$, Fig. 4A,B). Thus, limb withdrawals occurred at a shorter latency and consequently a lower temperature as determined by direct measurements of the glass over time. All $CGRP_{8-37}$ doses changed the forelimb responses such that paw withdrawal latencies for the hemisected group were statistically significant compared to baseline and the control group. The 50 nM dose of $CGRP_{8-37}$ raised the latency above baseline to 4.0 ± 1.5 s, a response consistent with hypoalgesia. For the hindlimbs, the 10 and 50 nM doses resulted in responses that were not statistically significantly different from pre-surgical baselines or control values with the 50 nM dose having the greater efficacy with response times of −4.9 ± 3.9 and 1.0 ± 2.5 s, respectively. Post-drug tests were not statistically different compared to post-surgical values for the hemisected group. The responses were correlated with dose for both forelimbs and hindlimbs, indicating a dose-dependent response as determined by Pearson product moment correlation statistics ($P < 0.05$). By contrast, the sham control group values were not statistically significant compared to pre-surgical values at all doses tested. As a control for the specificity of $CGRP_{8-37}$ in the observed response, a group of hemisected animals ($N = 5$) was tested after mechanical and thermal allodynia was established with a combination of 5 μl of 100 nM $CGRP_{8-37}$ and 5 μl of 1000 nM CGRP. For the thermal behavior tests, the change in foot withdrawal latency during the period of $CGRP_{8-37}$/CGRP effect was not statistically significantly different compared to uninjected values with a change in paw withdrawal latency of −4.0 ± 1.8 and −6.04 ± 1.3 s for forelimb and hindlimb, respectively.

4. Discussion

The data presented in this study support the hypothesis that the CGRP receptor antagonist, $CGRP_{8-37}$, attenuates both mechanical and thermal allodynia that occurs bilaterally in both forelimbs and hindlimbs after spinal hemisection in a spinal hemisection model of chronic central pain (Christensen et al., 1996a). $CGRP_{8-37}$ initiated a return to pre-surgical baseline levels for the mechanical and thermal responses tested in this study that was dose-dependent, i.e. the greater the $CGRP_{8-37}$ concentration the closer the responses were to baseline values. The same doses produced no significant changes in responses in the sham control group, or in both groups in responses to noxious stimuli or in responses to locomotor tests. These data suggest that CGRP plays an important role in the altered nociceptive neurotransmission after spinal cord injury under conditions that are not sedative. To our knowledge, this is the first direct demonstration of CGRP involvement in behavioral

changes in a chronic central neuropathic pain model. CGRP is thought to be involved in normal nociception (Yu et al., 1994) and a variety of other pain models, such as joint inflammation (Neugebauer et al., 1996; Schaible, 1996), dermal, intradermal or subcutaneous inflammation (Galeazza et al., 1995; Seybold et al., 1995; Malcangio and Bowery, 1996; Löfgren et al., 1997) and peripheral nerve injury (Miki et al., 1998). Therefore, it can be concluded that CGRP plays a role in both normal and abnormal nociceptive transmission.

In contrast to the studies in which injection of $CGRP_{8-37}$ produced anti-nociceptive effects in normal rats on the hot plate and paw pressure tests (Yu et al., 1994), our sham control rats showed no significant changes in response to mechanical or thermal behavioral tests at the doses of $CGRP_{8-37}$ tested, which is consistent with results in normal mice using the tail flick test (Saxen et al., 1994). Our results are consistent with previous studies in which intrathecal co-injections of CGRP and $CGRP_{8-37}$ did not inhibit the flexor reflex in acutely decerebrate rats suggesting that the spinal facilitatory effect of CGRP is not antagonized by $CGRP_{8-37}$ in the non-neuropathic spinal circuit (Xu and Wiesenfeld-Hallin, 1996). Differences in the studies may be attributed to differences in species, doses and route of delivery. However, we interpret our data to indicate that the spinal hemisection resulted in a chronic change in CGRP receptor activation such that the same doses that are necessary to attenuate the allodynia responses in the chronic pain model are not effective in altering the responses to both mechanical and thermal stimuli in the sham control group. This suggests that the CGRP receptor activation is greater in animals from the spinal hemisection group and that similar CGRP receptor activation does not occur in the transmission of nociception through normal spinal pathways. One mechanism that could account for an increased receptor activation would be an increase in the amount of transmitter released during somatosensory stimulation of rats in the spinal hemisected group compared to the sham control group.

Evidence of an increase in CGRP protein expression and distribution bilaterally in the low thoracic and lumbar dorsal horn after spinal hemisection or spinal transection has been reported (Christensen and Hulsebosch, 1997b; Krenz and Weaver, 1998; Krenz et al., 1999). CGRP is normally found in laminae I and II; however, CGRP distribution increased after thoracic spinal hemisection and transection into deeper dorsal horn laminae, laminae III and IV (Christensen and Hulsebosch, 1997b; Krenz and Weaver, 1998; Krenz et al., 1999), which is where the projections of primary afferents associated with touch, pressure and kinesthesia are classically held to project (Willis and Coggeshall, 1991). These data taken together with previous ultrastructural studies demonstrating an increase in the number of centrally projecting fine primary afferent fibers after spinal hemisection (Hulsebosch and Coggeshall, 1983) and an increase in the number of CGRP containing synaptic terminals after spinal deafferentation (McNeill et al., 1988, 1991) compared to sham control values suggest that CGRP spinal circuits subserving nociception are altered after spinal injury.

One predicted result of an increased CGRP projection pattern would be that an incoming peripheral volley would be amplified by the increased number of central projections and synapses. The mechanistic basis for this would be comparable to wind-up. Wind-up is defined as an increased responsiveness of dorsal horn neurons by repetitive C-fiber stimulation (Mendell, 1966). For example, in the case of an increased number of C-fiber central terminals onto a single dorsal horn neuron from a single C-fiber, an incoming peripheral volley could produce one mechanism for the permanent equivalent of wind-up, with resultant central sensitization. In normal spinal circuits, the increased responsiveness due to wind-up lasts for several minutes and is dependent on activation of NMDA receptors (Davies and Lodge, 1987; Dickenson and Sullivan, 1987, 1990; Wilcox, 1993). CGRP is known to stimulate the release of aspartate and glutamate (Kangrga et al., 1990), which act on the NMDA receptor and this finding is not modified by capsaicin. Thus, Kangrga et al. (1990) suggest that the observed CGRP stimulated increases in aspartate and glutamate are not from primary afferent sources of EAA but are achieved through activation of CGRP receptors on EAA interneurons. EAA synapses, of course, are found on spinal thalamic tract (STT) cells (Willis and Coggeshall, 1991). It has been demonstrated that increased primary C-fiber input can result in mechanical allodynia to touch and is NMDA-dependent (Ma and Woolf, 1995). Since the majority of C-fibers contain CGRP, it is not unreasonable to hypothesize that the CGRP receptor may be indirectly involved in the observed response of the Ma and Woolf report. In this case, a peripheral stimulus which results in increased responsiveness after a manipulation (as in the case of the hemisection model) might be blocked by an antagonist at the level of primary afferent to interneuron in the spinal circuit. Additionally, CGRP terminals project directly onto STT cells in the monkey (Carlton et al., 1990) and can directly influence STT excitability by an increased number of terminal projections as could occur with CGRP sprouting. Thus, both indirect and direct STT circuit changes can provide an alternate, non-exclusive mechanism for peptidergic involvement in maintained central sensitization of dorsal horn neurons.

The mechanism of CGRP action is not clear. The CGRP receptor exists in several forms (Chatterjee and Fisher, 1991; Gray et al., 1991; Poyner, 1992) and there is evidence for both pre-synaptic and post-synaptic receptor location (Oku et al., 1987; Ryu et al., 1988; Poyner, 1992; Yu et al., 1994) which would affect its mode of action. CGRP produces delayed, but prolonged, depolarization in single cell recordings of dorsal horn neurons, even in the presence of tetrodotoxin (Ryu et al., 1988). Additional evidence of CGRP involvement in nociception, although indirect, is based on studies of the effects of CGRP antiserum (anti-

CGRP). Anti-CGRP increased the nociceptive threshold in both normal rats and adjuvant-induced arthritic rats (Kuraishi et al., 1988), increased the paw withdrawal latency in adjuvant inflamed rats (Kawamura et al., 1989) and alleviated cold stress-induced hyperalgesia in rats (Satoh et al., 1992). It is of interest to note that the normal, noxious stimulus response was not altered by the CGRP receptor antagonist as evidenced by the absence of change in response to the pinprick in the present study. Thus, while $CGRP_{8-37}$ administration at the doses tested reduced the abnormal mechanical allodynia, these doses did not influence the number of withdrawals to pinprick stimulation for either the experimental or the control animals. Thus, it may be that only the polymodal nociceptive primary afferent population and not the high or low threshold nociceptive population responds by an increased CGRP release after spinal injury-induced reorganization.

One possible interpretation is that the behavioral responses are not directly a result of $CGRP_{8-37}$ injections, but an indirect effect due to the stress of an injected peptide in solution. There are several lines of evidence that do not support this interpretation. The physical properties of the test doses in terms of the pH value were the same while the effect of a test peptide would be negligible unless it is highly selective as is the case for the selective CGRP antagonist, $CGRP_{8-37}$ (Jansen-Olesen et al., 1996; Perren et al., 1996; Kagstrom and Holmgren, 1998). The best vehicle addition would be an inactive isomer; however, inactive isomers are not available for many compounds, including the $CGRP_{8-37}$. Furthermore, the animals receiving 10 μl of vehicle injection would receive the same general 'stress' as the animals receiving 10 μl of a nM solution of a peptide in vehicle, which could be either hypersensitivity or analgesia. If the peptide induced stress, then both the sham control and the spinal hemisected groups would behave similarly (either hypersensitivity or analgesia). Our data did not support a change in data that was related to 'stress' due to peptide injection alone since only the spinally hemisected group, and not the sham control group, demonstrated behavioral changes in response to the $CGRP_{8-37}$. Along this line of reasoning, both groups demonstrated no significant behavioral changes at all to vehicle injections compared to pre-injection behavior or post-injection behavior, which would seem to rule out effects of stress on the behavioral measures tested that might result from 10 μl of volume injected into the intrathecal space. Finally, the most compelling fact supporting the specificity of the $CGRP_{8-37}$ behavioral results was the neutralization of $CGRP_{8-37}$ by a 10-fold excess of CGRP that prevented the attenuation of allodynia.

Other possible anti-nociceptive mechanisms are indirect mechanisms such as those through other transmitter pathways. For example, CGRP is thought to interact with the substance P (SP) neurotransmitter and prolong the action of SP. Specifically, capsaicin treatment stimulates release of SP from the dorsal spinal cord. While CGRP alone does not stimulate SP release, CGRP given just prior to capsaicin treatment potentiates the SP release, producing greater SP levels than capsaicin alone (Oku et al., 1987). Additionally, intrathecal (i.t.) CGRP delivery just prior to i.t. SP delivery produces a more intense decrease in withdrawal latency than SP alone in the tail flick test (Cridland and Henry, 1989). CGRP's modulatory effects on responses to SP have been demonstrated at the level of single cell recordings (Biella et al., 1991), in the flexion withdrawal reflex (Woolf and Wiesenfeld-Hallin, 1986), and in nociceptive behaviors such as self-directed biting (Wiesenfeld-Hallin et al., 1984). The method by which CGRP alters the SP signal has not been defined, but evidence suggests that CGRP inhibits the enzymatic breakdown of SP to $SP_{(1-7)}$ (Le Grevès et al., 1985), since the same endopeptidases degrade both SP and CGRP (Chen et al., 1996). Excess CGRP would inhibit degradation of SP, thereby potentiating the effects of SP in the synaptic cleft (Le Grevès et al., 1985).

In addition to SP, other transmitter systems may be involved. CGRP action may include the excitatory amino acids which are associated with nociceptive spinal circuits (Willis and Coggeshall, 1991). For example, CGRP stimulates the release of aspartate and glutamate, which are excitatory amino acids involved in nociceptive circuits (Kangrga et al., 1990). Additionally, recent work suggests that CGRP receptor activation is influenced by opioid levels (Ménard et al., 1995; Yu et al., 1996) and CGRP release is influenced by cytokines, with release inhibited by at least one cytokine, interleukin-1β (Malcangio et al., 1996). Finally, CGRP receptors in spinal cord cultures of neurons and of astrocytes appear to be coupled to adenyl cyclase by G-proteins which induce the formation of cAMP and, at high doses, to activate guanyl cyclase to form cGMP; however, they have no effect on the formation of inositol phosphates (Parsons and Seybold, 1997). Thus, second messenger systems in both neuronal and glial cells are activated by CGRP which can act by increasing the excitability of dorsal horn neurons (Parsons and Seybold, 1997), providing a basis for central sensitization (Neugebauer et al., 1996) which is known to occur after spinal hemisection (Christensen and Hulsebosch, 1997a).

It is possible that CGRP's action as a primary modulator of nociception may involve the development of sensitization rather than short-lived (millisecond) excitation of spinal circuits that is related to dose, species or the absence or presence of inflammation or of neuropathy. For example, CGRP when injected in pmol doses intrathecally in mice did not produce any obvious pain-related behaviors such as self-directed biting, scratching, or grooming (Gamse and Saria, 1986), although we found that a single 10 μl intrathecal injection of 100 nM of CGRP into adult rats produced writhing, spontaneous biting and vocalization (Hulsebosch, unpublished data). Multiple 0.1 fmol doses of CGRP injections peripherally into the hindpaw over 3 h produced a hyperalgesic response (Nakamura-Craig and Gill, 1991). In other experiments, adjuvant induced inflammation of the hind paw led to an increase in CGRP in the sciatic

nerve on day 5 (Donnerer and Stein, 1992). Finally, several spinal cord lesion studies demonstrate increased CGRP-like immunoreactivity in the dorsal horn that persists for several months after injury (Christensen and Hulsebosch, 1997b). While CGRP's role, acutely, may be limited to a supportive function for SP, our data support the hypothesis that CGRP plays a greater role in chronic central neuropathy, perhaps by chronic sensitization of dorsal horn nociceptive neurons (Christensen and Hulsebosch, 1997a).

In summary, chronic central pain syndromes are characterized by the presence of persistent pain (Vierck, 1991; Lenz et al., 1994) with concomitant changes in peripheral somatosensory responses (Vierck, 1991). The data in the present manuscript demonstrate behavior consistent with a decrease in mechanical and thermal allodynia in response to peripheral stimuli after intrathecal administration of $CGRP_{8-37}$. Intrathecal administration of $CGRP_{8-37}$ at the 50 nM dose alleviates the abnormal nociceptive state in both the forelimbs and the hindlimbs, indicating a primary role for CGRP in this model of chronic central pain. Thus, since the present study demonstrates decreased abnormal somatosensory responses to peripheral stimuli after $CGRP_{8-37}$ administration, we conclude that this agent may be efficacious in the treatment of chronic central pain syndromes.

Acknowledgements

The authors wish to thank Ms. Debbie Pavlu for her excellent secretarial assistance. This study was supported by Mission Connect, The Institute for Rehabilitation and Research in Houston, the Spinal Cord Research Foundation and NIH grant NS 11255.

References

Amara SG, Jonas V, Rosenfeld MG, Ong ES, Evans RM. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. Nature 1982;298:240–244.

Amara SG, Arriza JL, Leff SE, Swanson LW, Evans RM, Rosenfeld MG. Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene-related peptide. Science 1985;229: 1094–1097.

Basso M, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma 1995;12:1–21.

Bennett GJ, Xie YK. A peripheral nonneuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 1988;33:87–107.

Biella G, Panara C, Pecile A, Sotgiu ML. Facilitatory role of calcitonin gene-related peptide (CGRP) on excitation induced by substance P (SP) and noxious stimuli in rat spinal dorsal horn neurons. An iontophoretic study in vivo. Brain Res 1991;559:352–356.

Cameron AA, Leah JD, Snow PJ. The coexistence of neuropeptides in feline sensory neurons. Neuroscience 1988;27:969–979.

Carlton SM, Westlund KN, Zhang D, Sorkin L, Willis WD. Calcitonin gene-related peptide containing primary afferent fibers synapse on primate spinothalamic tract cells. Neurosci Lett 1990;109:76–81.

Chatterjee TK, Fisher RA. Multiple affinity forms of the calcitonin gene-related peptide receptor in rat cerebellum. Mol Pharmacol 1991;39:798–804.

Chen JJ, Barber LA, Dymshitz J, Vaski MR. Peptidase inhibitors improve recovery of substance P and calcitonin gene-related peptide release from rat spinal cord slices. Peptides 1996;17:31–37.

Chiba T, Yamaguchi A, Yamatani T, Naksmura A, Morishita T, Inui T, Fukase M, Noda T, Fujita T. Calcitonin gene-related peptide antagonist human CGRP(8-37). Am J Physiol 1989;256:E331–E335.

Choi C, Yoon Y, Na H, Kim S, Chung JM. Behavioral signs of ongoing pain and cold allodynia in a rat model of neuropathic pain. Pain 1994;59: 369–376.

Christensen MD, Everhart AW, Pickelman JT, Hulsebosch CE. Mechanical allodynia and thermal hyperalgesia in chronic central pain following spinal cord injury. Pain 1996a;68:97–107.

Christensen MD, Supowit SC, DiPette DJ, Hulsebosch CE. Enhanced gene expression of CGRP and GAP-43 in dorsal root ganglia by spinal hemisection. J Neurotrauma 1996b;13:602.

Christensen MD, Hulsebosch CE. Chronic central pain after spinal cord injury. J Neurotrauma 1997a;14:517–537.

Christensen MD, Hulsebosch CE. Spinal cord injury and anti-NGF treatment results in changes in CGRP density and distribution in the dorsal horn in the rat. Exp Neurol 1997b;147:463–475.

Chung K, Lee WT, Carlton SM. The effects of dorsal rhizotomy and spinal cord isolation on calcitonin gene related peptide labeled terminals in the rat lumbar dorsal horn. Neurosci Lett 1988;90:27–32.

Cridland RA, Henry JL. Effects of intrathecal administration of neuropeptides on spinal nociceptive reflex in the rat: VIP, galanin, CGRP, TRH, somatostatin and angiotensin II. Neuropeptides 1988;11:23–32.

Cridland RA, Henry JL. Intrathecal administration of CGRP in the rat attenuates a facilitation of the tail flick reflex induced by either substance P or noxious cutaneous stimulation. Neurosci Lett 1989;102:241–246.

Davies SN, Lodge D. Evidence for involvement of N-methylaspartate receptors in wind-up of class 2 neurons in the dorsal horn of the rat. Brain Res 1987;424:402–406.

Dickenson AH, Sullivan AF. Evidence for a role of the NMDA receptor in the frequent dependent potentiation of deep rat dorsal horn nociceptive neurones following C fibre stimulation. Neuropharmacology 1987;26:1235–1238.

Dickenson AH, Sullivan AF. Differential effects of excitatory amino acid antagonists on dorsal horn nociceptive neurones in the rat. Brain Res 1990;506:31–39.

Donnerer J, Stein C. Evidence for an increase in the release of CGRP from sensory nerves during inflammation. Ann N Y Acad Sci 1992;657:505–506.

Galeazza MT, Garry MG, Yost HJ, Strait KA, Hargreaves KM, Seybold VS. Plasticity in the synthesis and storage of substance P and calcitonin gene-related peptide in primary afferent neurons during peripheral inflammation. Neuroscience 1995;66:443–458.

Gamse R, Saria A. Nociceptive behavior after intrathecal injections of substance P, neurokinin A and calcitonin gene-related peptide in mice. Neurosci Lett 1986;70:143–147.

Gibson SJ, Polak JM, Bloom SR, Sabate IM, Mulderry PM, Ghatei MA, McGregor GP, Morrison JF, Kelly JS, Evans RM. Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and of eight other species. J Neurosci 1984;4:3101–3111.

Gray DW, Marshall I, Bose C, Foulkes R, Hughes B. Subtypes of calcitonin gene related peptide receptor in vascular tissues. Br J Pharmacol 1991;102:189.

Hargreaves K, Dubner R, Brown F, Flores D, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 1988;32:77–88.

Hökfelt T, Arvidsson U, Ceccatelli S, Cortes R, Cullheim S, Dagerlind A, Johnson H, Orazzo C, Piehl F, Pieribone V, Schalling M, Terenius L, Ulfhake B, Verge VM, Villar M, Wiesenfeld-Hallin Z, Xu XJ, Zhang X. Calcitonin gene-related peptide in the brain, spinal cord and some peripheral systems. Ann N Y Acad Sci 1992;657:119–133.

Hulsebosch CE, Coggeshall RE. A comparison of axonal numbers in dorsal roots following spinal cord hemisection in neonate and adult rats. Brain Res 1983;265:187–197.

Ishida-Yamamoto A, Tohyama M. Calcitonin gene-related peptide in the nervous tissue. Prog Neurobiol 1989;33:335–386.

Jansen-Olesen I, Mortensen A, Edvinsson L. Calcitonin gene-related peptide is released from capsaicin-sensitive nerve fibres and induces vasodilation of human cerebral arteries concomitant with activation of adenylyl cyclase. Cephalagia 1996;16:310–316.

Kagstrom J, Holmgren S. Calcitonin gene-related peptide (CGRP), but not tachykinins, causes relaxation of small arteries from the rainbow trout gut. Peptides 1998;19:577–584.

Kangrga I, Larew JSA, Randic M. The effects of substance P and calcitonin gene-related peptide on the efflux of endogenous glutamate and aspartate from the rat spinal dorsal horn in vitro. Neurosci Lett 1990;108:155–160.

Kawamura M, Kuraishi Y, Minami M, Satoh M. Antinociceptive effect of intrathecally administered antiserum against calcitonin gene-related peptide on thermal mechanical noxious stimuli in experimental hyperalgesic rats. Brain Res 1989;497:199–203.

Krenz NR, Weaver LC. Sprouting of primary afferent fibers after spinal cord transection in the rat. Neuroscience 1998;85:443–458.

Krenz NR, Meakin SO, Krassioukov AV, Weaver LC. Neutralizing intraspinal nerve growth factor blocks autonomic dysreflexia caused by spinal cord injury. J Neurosci 1999;19:7405–7414.

Kuraishi Y, Nanayama T, Ohno H, Minami M, Satoh M. Antinociception induced in rats by intrathecal administration of antiserum against calcitonin gene-related peptide. Neurosci Lett 1988;92:325–329.

Le Grevès P, Nyberg F, Terenius L, Hökfelt T. Calcitonin gene-related peptide is a potent inhibitor of substance P degradation. Eur J Pharm 1985;115:309–311.

Lenz FA, Kwan HC, Martin R, Tasker R, Richardson RT, Dostrovsky JO. Characteristics of somatotopic organization and spontaneous neuronal activity in the region of the thalamic principal sensory nucleus in patients with spinal cord transection. J Neurophysiol 1994;72:1570–1587.

Löfgren O, Yu LC, Theodorsson E, Hansson P, Lundeberg T. Intrathecal $CGRP_{8-37}$ results in a bilateral increase in hindpaw withdrawal latency in rats with a unilateral thermal injury. Neuropeptides 1997;31:601–607.

Ma Q-P, Woolf CJ. Noxious stimuli induce an N-methyl-d-aspartate receptor-dependent hypersensitivity of the flexion withdrawal reflex to touch: implication for the treatment of mechanical allodynia. Pain 1995;61:383–390.

Malcangio M, Bowery NG. Calcitonin gene-related peptide content, basal outflow and electrically-evoked release from monoarthritic rat spinal cord in vitro. Pain 1996;66:351–358.

Malcangio M, Bowery NG, Flower RJ, Perretti M. Effect of interleukin-1β on the release of substance P from rat isolated spinal cord. Eur J Pharm 1996;299:113–118.

Marti E, Gibson SJ, Polak JM, Facer P, Springall DR, Van Aswegen G, Aitchison M, Koltzenburg M. Ontogeny of peptide- and amine-containing neurones in motor, sensory and autonomic regions of rat and human spinal cord, dorsal root ganglia and rat skin. J Comp Neurol 1987;266:332–359.

McNeill DL, Chung K, Carlton SM, Coggeshall RE. Calcitonin gene-related peptide immunostained axons provide evidence for fine primary afferent fibers in the dorsal and dorsolateral funiculi of the rat spinal cord. J Comp Neurol 1988;272:303–308.

McNeill DL, Carlton SM, Hulsebosch CE. Intraspinal sprouting of calcitonin gene-related peptide containing primary afferents after deafferentation in the rat. Exp Neurol 1991;114:321–329.

Ménard DP, van Rossum D, Kar S, Quirion R. Alteration of calcitonin gene related peptide and its receptor binding sites during the development of tolerance to μ and δ opioids. Can J Physiol Pharmacol 1995;73:1089–1095.

Mendell LM. Physiological properties of unmyelinated fiber projection to the spinal cord. Exp Neurol 1966;16:316–322.

Mercadante S. Problems of long-term spinal opioid treatment in advanced cancer patients. Pain 1999;79:1–13.

Miki K, Fukuoka T, Tokunaga A, Noguchi K. Calcitonin gene-related peptide increase in the rat spinal dorsal horn and dorsal column nucleus following peripheral nerve injury: upregulation in a subpopulation of primary afferent sensory neurons. Neuroscience 1998;82:1243–1252.

Mulderry PK, Ghatei MA, Spokes RA, Jones PM, Pierson AM, Hamid QA, Kanse S, Amara SG, Burrin JM, Legon S, Polak JM, Bloom SR. Differential expression of α-CGRP and β-CGRP by primary sensory neurones and enteric autonomic neurones of the rat. Neuroscience 1988;25:195–205.

Nakamura-Craig M, Gill BK. Effect of neurokinin A, substance P and calcitonin gene related peptide in peripheral hyperalgesia in the rat paw. Neurosci Lett 1991;124:49–51.

Neugebauer V, Rumenapp P, Schaible H-G. Calcitonin gene-related peptide is involved in the spinal processing of mechanosensory input from the rat's knee joint and in the generation and maintenance of hyperexcitability of dorsal horn neurons during development of acute inflammation. Neuroscience 1996;71:1095–1109.

Oku R, Satoh M, Fujii N, Otaka A, Yajima H, Takagi H. Calcitonin gene-related peptide promotes mechanical nociception by potentiating release of substance P from the spinal dorsal horn in rats. Brain Res 1987;403:350–354.

Parsons AM, Seybold VS. Calcitonin gene-related peptide induces the formation of second messengers in primary cultures of neonatal rat spinal cord. Synapse 1997;26:235–242.

Perren MJ, Connor HE, Beattie DT. NK1 and CGRP receptor-mediated dilation of the carotid arterial bed of the anaesthetized rabbit. Neuropeptides 1996;30:141–148.

Poyner DR. Calcitonin gene-related peptide: multiple actions, multiple receptors. Pharmacol Ther 1992;56:23–51.

Rosenfeld MG, Mermod JJ, Amara SG, Swanson LW, Sawchenko PE, Rivier J, Valse WW, Evans RM. Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. Nature 1983;304:129–135.

Ryu PD, Gerber G, Murase KM, Randic M. Actions of calcitonin gene-related peptide on spinal dorsal horn neurons. Brain Res 1988;441:357–361.

Satoh M, Kuraishi Y, Kawamura M. Effects of intrathecal antibodies to substance P, calcitonin gene-related peptide and galanin on repeated cold stress-induced hyperalgesia: comparison with carrageenan-induced hyperalgesia. Pain 1992;49:273–278.

Saxen MA, Smith FL, Dunlow LD, Dombrowski DS, Welch SP. The hypothermic and antinociceptive effects of intrathecal injection of CGRP(8-37) in mice. Life Sci 1994;55:1665–1674.

Schaible H-G. On the role of tachykinins and calcitonin gene-related peptide in the spinal mechanisms of nociception and in the induction and maintenance of inflammation-evoked hyperexcitability in spinal cord neurons (with special reference to nociception in joints). In: Kumazawa T, Kruger L, Mizumura K, editors. Progress in brain research, 113. Amsterdam: Elsevier, 1996. pp. 423–441.

Senba E, Tohyama M. Calcitonin gene-related peptide containing autonomic efferent pathways to the pelvic ganglia of the rat. Brain Res 1988;449:386–390.

Seybold VS, Galeazza MT, Garry MG, Hargreaves KM. Plasticity of calcitonin gene related peptide neurotransmission in the spinal cord during peripheral inflammation. Can J Physiol Pharmacol 1995;73:1007–1014.

Tarlov IM, Klinger H. Spinal cord compression studies. II. Time limits for recovery after acute compression in dogs. Arch Neurol Psych 1954;71:271–290.

Van Rossum D, Ménard DP, Fournier A, St-Pierre S, Quirion R. Binding profile of a selective calcitonin gene-related peptide (CGRP) receptor antagonist ligand $[^{125}$I-Tyr]$hCGRP_{8-37}$ in rat brain and peripheral tissues. J Pharm Exp Ther 1994;269:846–853.

Vierck Jr. CJ. Can mechanisms of central pain syndromes be investigated in animal models? In: Casey KL, editor. Pain and central nervous system

disease: the central pain syndromes, New York: Raven Press, 1991. pp. 129–141.

Wiesenfeld-Hallin Z, Hökfelt T, Lundberg JM, Forssmann WG, Reinecke M, Tschopp FA, Fischer JA. Immunoreactive calcitonin gene-related peptide and substance P coexist in sensory neurons to the spinal cord and interact in spinal behavioral responses of the rat. Neurosci Lett 1984;52:199–204.

Wilcox GL. Spinal mediators of nociceptive neurotransmission and hyperalgesia. APS J 1993;2:265–275.

Willis WD, Coggeshall RE. Sensory mechanisms of the spinal cord, New York: Plenum, 1991. pp. 575.

Woolf C, Wiesenfeld-Hallin Z. Substance P and calcitonin gene-related peptide synergistically modulate the gain of the nociceptive flexor withdrawal reflex in the rat. Neurosci Lett 1986;66:226–230.

Xu X-J, Wiesenfeld-Hallin Z. Calcitonin gene-related peptide (8-37) does not antagonize calcitonin gene-related peptide in rat spinal cord. Neurosci Lett 1996;204:185–188.

Yu L-C, Hansson P, Lundeberg T. The calcitonin gene-related peptide antagonist $CGRP_{8-37}$ increases the latency to withdrawal responses in rats. Brain Res 1994;653:223–230.

Yu L-C, Hansson P, Lundeberg T. The calcitonin gene-related peptide antagonist $CGRP_{8-37}$ increases the latency to withdrawal responses bilaterally in rats with unilateral experimental mononeuropathy, an effect reversed by naloxone. Neuroscience 1996;71:523–531.