# Nociception in Persistent Pancreatitis in Rats

## Effects of Morphine and Neuropeptide Alterations

*Louis P. Vera-Portocarrero, B.S.,* Ying Lu, M.D.,† Karin N. Westlund, Ph.D.,‡*

*Background:* Most animal models of pancreatitis are short-lived or very invasive. A noninvasive animal model of pancreatitis developed in highly inbred rats by Merkord *et al.* with symptoms persisting for 3 weeks was adopted in the current study to test its validity as a model of visceral pain in commercially available rats.

*Methods:* The persistent pancreatitis model was established by tail vein injection of dibutyltin dichloride. Animals were given 10% alcohol in their drinking water to enhance the pancreatitis attack. Blood serum pancreatic enzymes and nociceptive state were monitored for 3 weeks after dibutyltin dichloride or vehicle. Behavioral testing included reflexive withdrawal to mechanical and thermal stimulation of the abdominal area. The effect of morphine on nociceptive behaviors was tested. Histologic analysis of the pancreas and immunohistochemical analysis of substance P and calcitonin gene-related peptide in the spinal cord are included in the study.

*Results:* Compared with naïve and vehicle-only injected control groups, rats receiving dibutyltin dichloride demonstrated an increase in withdrawal events after von Frey stimulation and decreased withdrawal latency after thermal stimulation, signaling a sensitized nociceptive state through 7 days. These pain-related measures were abrogated by morphine. Blood serum concentrations of amylase and lipase as well as tissue inflammatory changes and substance P were also significantly elevated during this same time period.

*Conclusions:* These results indicate that animals with the dibutyltin dichloride–induced experimental pancreatitis expressed serum, histologic, and behavioral characteristics similar in duration to those present during acute attacks experienced by patients with chronic pancreatitis. These findings and responsivity to morphine suggest the utility of this model developed in a commercially available strain of rats for study of persistent visceral pain.

VISCERAL pain is frequently resistant to treatment by physicians, as in persons with chronic pancreatitis or pancreatic cancer.[1] Pain is the only sensation that can be

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* Graduate Assistant.    † Assistant Professor.    ‡ Professor.

Address reprint requests to Dr. Westlund: Department of Anatomy and Neurosciences, University of Texas Medical Branch, Medical Research Building, Room 10.138D, 301 University Boulevard, Galveston, Texas 77555-1043. Address electronic mail to: kwhigh@utmb.edu. Individual article reprints are available through the Journal Web site, www.anesthesiology.org.

elicited from the pancreas and occurs when the pancreas is inflamed or during pancreatic cancer.[2] Animal models of acute pancreatitis are abundant, but they represent acute noxious stimulation of the pancreas that resolves after a finite and typically short amount of time.[3–5] These models are valuable for the study of the acute events, but a model of longer duration evoking central sensitization and pain[6] is desirable for more clinically relevant study since it is likely that long-term changes are induced by the continuous barrage of noxious information from the periphery.

Nociceptive reflexive behaviors have been adopted as indicators of the pain state of an animal. Validation of specific behaviors is necessary in the establishment of animal models of painful conditions.[7] It is also important to elucidate the neurochemical expression changes notable during the generation and maintenance of central changes leading to those behaviors for the purpose of identifying targets for possible pharmacologic interventions.

In the human condition, pain elicited from the pancreas is often "referred" to the upper (epigastric) abdominal area and radiates to the back.[8,9] In human patients, these dermatomal areas are usually tender to touch (referred mechanical allodynia).[9] In animal models, this phenomenon is also observed in rats, where visceral pain is characterized as a lowered threshold of response to mechanical stimulation of the abdominal areas. The abdominal areas are assumed to be zones of referred pain for the animal.[10–12] In the current study we used these behavioral paradigms to test the development of nociceptive behaviors in animals with persistent pancreatic inflammation and to determine if these behaviors are responsive to morphine treatment as in the human condition.[13] At the same time, we monitored pancreatic enzyme serum concentrations (amylase and lipase) as a clinical marker of pancreatitis.[14]

Neuropeptides known to be present in the afferent fibers innervating visceral organs have been characterized.[15–17] Substance P (SP) and calcitonin gene-related peptide (CGRP) are found in the majority of fibers innervating the pancreas.[18,19] Both SP and CGRP typically undergo dynamic changes in their expression during the induction and maintenance of pain states. During the development of monoarthirtis, expression of SP and CGRP is increased.[20, 21] In models of peripheral somatic inflammation, SP and CGRP present a wide spectrum of plasticity at the spinal cord and dorsal root ganglion

levels.[22-24] In visceral pain studies, SP and CGRP content is decreased in colonic afferents after inflammation and released in the mouse colon after capsaicin,[25,26] while in the spinal cord, SP and CGRP staining is increased after colon inflammation.[27] Elucidation of the expression changes that these neuropeptides undergo in this model of pancreatitis with persistent visceral pain are described here. A preliminary report of this study has been published in abstract form.[28]

## Materials and Methods

### Induction of Pancreatitis

All experiments were approved by the University of Texas Medical Branch Animal Care and Use Committee and adhered to the guidelines of this committee as well as the policies on the Ethical Treatment of Research Animals published by the International Association for the Study of Pain. Male Lewis-inbred rats (Harlan, Indianapolis, IN) weighed between 150 and 200 g at the onset of the study. The animals were maintained under standard conditions with two rats per cage and given Teklab diet 8626. This diet is low in soybean oil and was preferred in this study since it has been shown that diets high in soybean isolectin content can be antinociceptive.[29] Three experimental groups were compared in these studies, including rats with persistent experimental pancreatitis (n = 8), vehicle injection (n = 8), or naïve controls (n = 6). Pancreatitis was induced in these animals according to a protocol reported by Sparmann *et al.*[30] The dibutyltin dichloride (DBTC) was dissolved first in 100% ethanol (two parts) and then mixed with glycerol (three parts). The DBTC (8 mg/kg body weight; Aldrich Co., Milwaukee, WI) was injected into the tail vein of rats during methohexital anesthesia (40 mg/kg body weight) in a volume of 250 $\mu$l solvent. For the control groups, the animal were naïve or the tail veins were injected with the vehicle (ethanol:glycerol, 2:3) only. The rationale for this vehicle-injected group is to control for any nonspecific effects of the vehicle solution itself. The DBTC and vehicle-injected groups were maintained on 10% ethanol in their drinking water throughout the experiment to provide enhancement of irritation to the pancreas according to Merkord *et al.*[31] On days 3, 7, 10, 14, and 21, blood was drawn from the tail vein to measure clinical serum parameters indicative of pancreatitis (amylase and lipase blood concentrations). Amylase serum concentrations were assayed using the amylase infinity kit (Sigma, St. Louis, MO) with 4,6-ethylidene-(G$_7$) p-nitrophenyl, D-maltohepatosid as the substrate. Lipase serum concentrations were assayed using the Lipase-PS kit (Sigma) with triolein as a substrate. Animals were transcardially perfused with paraformaldehyde for histologic and immunohistochemical procedures at each of the above mentioned time points.

### Behavioral Procedures

Before mechanical and thermal sensitivity testing, all animals were environmentally acclimated to the clear Plexiglas cubicle testing apparatus for 4 h daily for 3 days. Baseline testing took place a day before induction of pancreatitis. Mechanical hypersensitivity in the abdominal area was quantified by measuring the number of withdrawal events (either abdominal withdrawal from the von Frey filament or consequent licking of the abdominal area, or whole body withdrawal) in response to normally innocuous or subthreshold mechanical stimuli.[32] The stimuli, applied to the abdominal area, consisted of von Frey filaments with bending forces of 4.78 and 9.96 mN, which are considered subthreshold stimuli, and with a von Frey filament with bending force of 204.1 mN, used as a suprathreshold stimulus. In human perceptual terms, these mechanical forces represent a subthreshold stimulus, a light touch, and a poke, respectively.[33] To perform this test, rats were placed inside Plexiglas boxes on an elevated, fine fiberglass screen mesh and acclimated for 60 min before testing, The von Frey filaments were applied from underneath through the mesh floor, to the abdominal area at different points on the surface. A single trial consisted of 10 applications of von Frey filaments applied once every 10 s to allow the animal to cease any response and return to a relatively inactive position. It is unlikely that behavioral sensitization will occur with the testing interval of 10 s or more. The mean occurrence of withdrawal events in each of the trials was expressed as the number of responses of 10 applications, where 0 indicates no withdrawal and 10 indicates the maximum number of withdrawals. Three trials were performed in each animal, and the withdrawal events were averaged to obtain a single value per rat per time point.

Thermal hyperalgesia was measured by the latency of withdrawal to thermal stimuli in the noxious range as previously described by Bennet and Xie[34] and Hargreaves *et al.*[35] Animals were placed in Plexiglas boxes on an elevated glass plate through which a high-intensity light beam was shone. A radiant heat stimulus was applied by concentrating a beam of light through a hole (1 × 1 cm) in the light box onto the abdominal area. The light beam and timer were immediately stopped when the animal withdrew, allowing the measurement of time between the start of the light beam and the withdrawal event. Pilot studies demonstrated that the abdominal skin of the rats is not separated from the glass surface more than 1 mm. These same pilot studies showed that the rate of heat increase within this distance does not vary significantly. Forty-five minutes was allowed between each trial, and three trials were averaged. A withdrawal event to radiant heat applied to the abdomen was defined as abdominal withdrawal (either abdominal musculature contraction or lifting of the abdomen through postural adjustment) accompanied by head turning to-

ward the stimuli and licking of the abdominal area. The experimenter was blinded to the type of treatment that the animals had received.

*Morphine Study*

All three groups of rats (n = 6 per group) received either morphine sulfate (Paddock Laboratories, Minneapolis, MN) using a cumulative systemic dose regimen of 1, 5, and 10 mg/kg intraperitoneally (lower abdominal area) or saline vehicle every 30 min. Mechanical and thermal hypersensitivity was evaluated after each dose, ending with the last test 30 min after the last dose (cumulative, 10 mg/kg; 1.5 h after the first dose). The experimenter performing the behavioral tests was unaware of which animals had received either morphine or saline injection. The effectiveness of morphine on mechanical and thermal hypersensitivity in animals with pancreatitis was tested in comparisons with responses of animals injected only with vehicle solution. The scores for responses to the three individual von Frey filaments were totaled, and the single value was plotted for simplicity of presentation.

*Histologic Procedures*

Pancreatic tissues were removed before transcardial perfusion with paraformaldehyde, and the tissue was placed in several 0.1-M phosphate buffered saline (PBS, pH 7.4) washes to remove blood elements. After the washes, pancreatic tissue was placed in 4% paraformaldehyde for 3 h, subsequently diced into smaller pieces, and kept in the fixative solution for 2 days. The tissue was transferred through graded ethanol solutions (80%, 95%, and 100% twice each). Pancreatic tissue was then transferred to xylene washes twice and placed in crucibles with a solution of xylene and melted paraffin (50:50 ×2), placed in 100% paraffin (×2), and placed the oven for 45 min (60°C). Tissue was placed in cassettes and embedded in paraffin. Paraffin blocks were cut in 8-$\mu$m sections and stained with hematoxylin and eosin. As a control for nonspecific effects of DBTC, liver tissue was also taken from these rats and processed in the same manner as described above. Methylene blue dye was injected transcardially into rats with pancreatic inflammation and control rats to establish sites of inflammation by gross visual inspection for plasma extravasation. The methylene blue (2%) was injected in four 1-ml boluses and allowed to circulate for 1 h before visual inspection.

*Immunohistochemical Procedures*

On days 3, 7, 14, and 21 after tail vein injection, Lewis rats from all three groups (naive, vehicle, and DBTC-treated, n = 4 per group per time point) were perfused, and the thoracic segments of the spinal cords were removed and processed for immunohistochemistry. Rats were perfused transcardially with 50 ml of heparinized saline at 37°C followed by 500 ml of cold (4°C) 4%

paraformaldehyde solution (pH 7.4). The spinal cords were removed carefully and postfixed in 4% paraformaldehyde solution at room temperature for 4 h before cryoprotection and storage in 30% sucrose–phosphate buffer solution overnight. The T6–T10 segments of the spinal cord were embedded in OCT cryoprotectant compound (Allegiance Healthcare Corp., Torrance, CA) and stored at −70°C until processed. Tissue blocks were cut in 30-$\mu$m-thick coronal sections on a freezing sliding microtome, and alternate sections were collected in PBS (pH 7.4). The free-floating sections were rinsed six times in the PBS used as the diluent buffer (0.1 M PBS) throughout the procedure before immersion in 3% normal goat serum–triton 100-X for 30 min at room temperature. The sections were incubated in either rabbit anti-SP (1:30,000; Incstar, Stillwater, MN) or rabbit anti-CGRP (1:5000; Peninsula, Belmont, CA) for 24 h, after which sections were rinsed in PBS and incubated in goat anti-rabbit immunoglobulin G (1:200, Vector Inc., Burlingame, CA) for 2 h. After rinsing in PBS, the sections were incubated in ExtrAvidin-Peroxidase–PBS (1:1000; Sigma) for 90 min. After rinsing in 0.1 M PBS for 30 min, the stain was visualized after reaction with 0.015% 3,3'-diaminobenzidine (Sigma) in phosphate buffer (pH 6.0, 0.1 M) containing 0.075% hydrogen peroxide. After six subsequent rinses in 0.1 M PBS, the sections were mounted onto gelatin-coated slides, air-dried, dehydrated in ascending concentrations of ethyl alcohol, cleared in xylene, and cover slipped with DPX mounting medium. As a negative staining control, sections were processed for immunohistochemistry with the primary antibody deleted. Immunoabsorption testing using these antibodies was recently reported by us.[27] All sections from all of the groups were processed at the same time with the same solutions in wire bottom trays, assuring that all tissues received similar exposure to all reagents. Stained sections were observed with a Nikon FXA microscope (Nikon, Melville, NY) linked to a Pentium PC (Intel, Santa Clara, CA) though an Optronics DEI-470 (Optronics, Goleta, CA) digitally enhanced color microscope video camera with built-in Digital Image Processor at ×10 magnification. The average immunodensity was measured using the NIH image analysis software (Scion Corporation, Frederick, MD). The average intensity of staining of lamina I and II in five random sections from each animal was determined by outlining the laminar borders and comparing the density of immunostaining relative to measurements obtained for white matter, which was used as a reference point to normalize staining among sections.

*Statistical Procedures*

All data were expressed as mean ± SEM. Behavioral and immunohistochemical data were tested for statistical significance with repeated-measures analysis of variance with a significance level set at $P < 0.05$ to allow multiple

comparisons between groups at different time points. *Post hoc* comparisons included Student paired and unpaired *t* tests to test statistical differences between control and experimental groups and before and after treatment comparison within the same group.

## Results

### Characterization of the Pancreatitis Model

To assess the inflammatory state of the pancreas, blood was drawn from the tail vein of Lewis rats treated either with DBTC or vehicle. Blood from animals in the naive group was also drawn for analysis. Serum was analyzed for pancreatic enzymes content (amylase and lipase). Rats with DBTC treatment had significantly elevated concentrations of amylase (fig. 1A) and lipase (fig. 1B) during the first week after tail vein injection. The steady increase of serum parameters in the DBTC group was not observed in the control groups.

Pancreatic samples taken from all rats were stained with hematoxylin and eosin and examined for evidence of inflammation. Figure 2A shows a histologic section from normal pancreas for comparison. Signs of acute pancreatitis included interstitial edema, moderate infiltration of neutrophilic granulocytes, and acinar atrophy (fig. 2B). As a control for specificity of the effects of DBTC, histologic samples from the liver were also examined. The hematoxylin–eosin staining for liver revealed no differences between control rats and rats treated with DBTC (figs. 2C and 2D, respectively). The abdominal wall and the internal organs were inspected visually at the end of the behavioral studies for presence of blue dye extravasation. No traces of blue dye were present on the abdominal wall or the other organs of the abdominal cavity. Extravasation was clearly evident, particularly associated with the head (duodenal portion) of the pancreas.

### Behavioral Studies

Lewis rats, which had received a tail vein injection of DBTC, vehicle injection, or no manipulation, were tested at 1, 3, 7, 10, 14, and 21 days after injection. The observer performing the behavioral testing was blind to the animal's condition. The rats injected with DBTC demonstrated increased sensitization to mechanically applied stimuli on the abdominal area, *i.e.*, the number of withdrawals from the von Frey stimulation at different gram forces increased (fig. 3). The percentage of animals that responded by withdrawing from the von Frey filaments in more than 2 of 10 applications increased with the force of the filaments. Thus, the 204.1-mN von Frey filament evoked responses of greater than 2 in 100% of the rats. DBTC-injected rats tested for thermal reactivity demonstrated increased sensitivity to heat stimuli on days 3 and 7 after injection compared with naive and
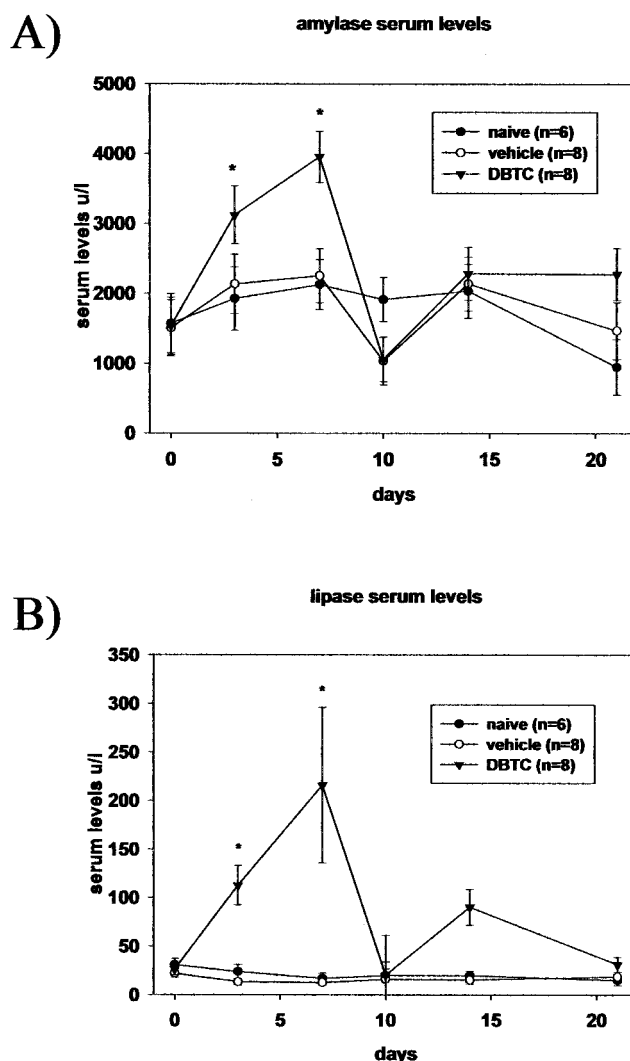


**Fig. 1. Measurement of serum concentrations of amylase and lipase from naive, vehicle-injected, and dibutyltin dichloride (DBTC)-injected rats. (*A*) Time course of amylase serum concentrations through 3 weeks after tail vein injection. \*$P < 0.05$ compared with vehicle-injected group. (*B*) Time course of lipase serum concentrations through 3 weeks after tail vein injection. \*$P < 0.05$ compared with vehicle-injected group. Concentrations are expressed in units per liter. Error bars denote SEM.**

vehicle control groups and compared with their own baseline (fig. 4). The withdrawal latencies to heat stimuli for DBTC-injected animals were significantly different from their own baselines on days 1, 3, and 7 (fig. 4). Rats were also tested on additional days in between time points 3 and 7 (von Frey and thermal stimuli). On these additional days, rats also demonstrated nociceptive behaviors that were similar to the ones reported at 3 and 7 days (data not shown). The effects of DBTC were less in Lewis-inbred rats that were fed a soy meal diet (data not shown).

### Effect of Morphine on Nociceptive Behavior

For this particular study, the nociceptive responses to each von Frey filament were summated to obtain one
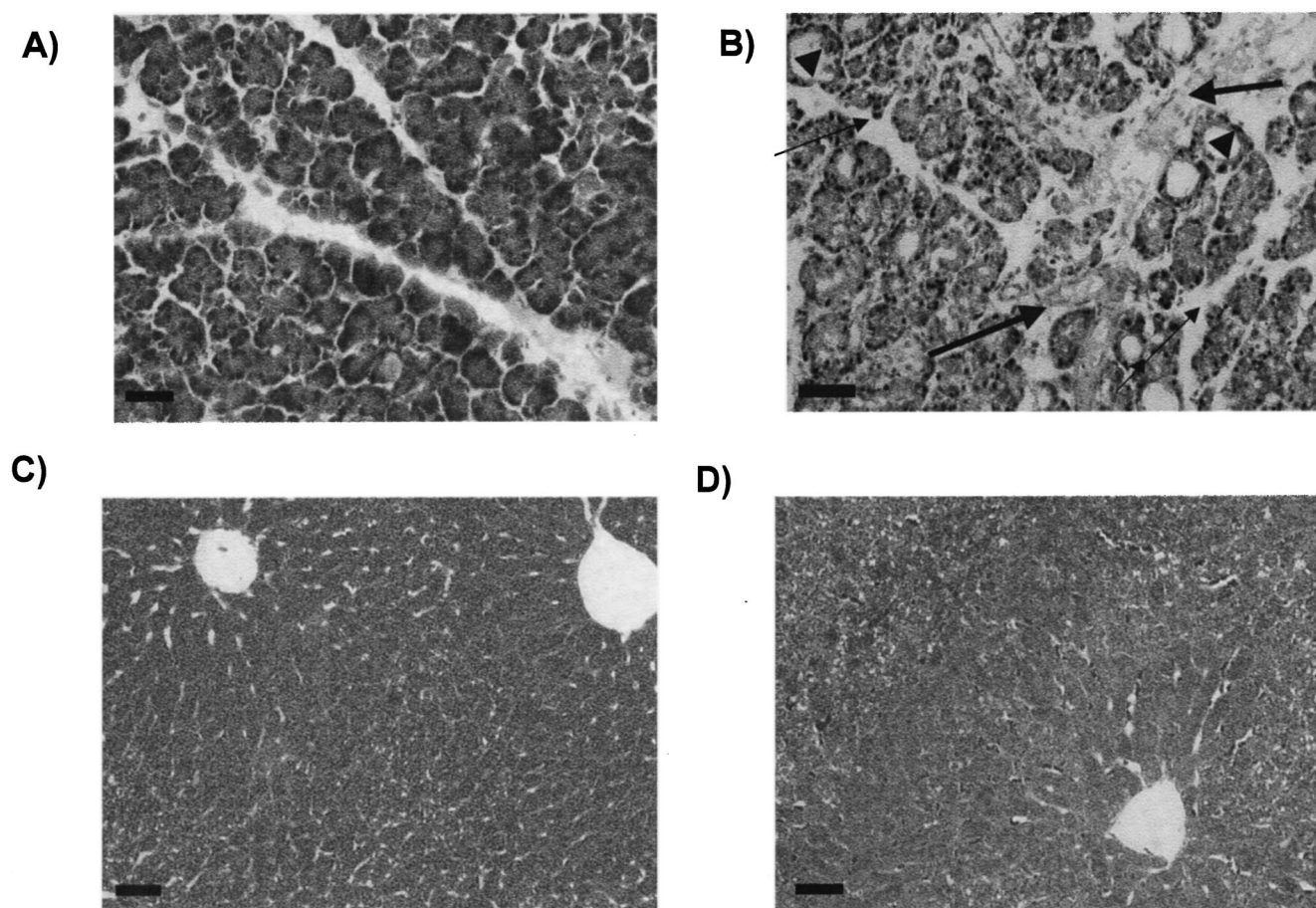
Fig. 2. Paraffin sections of pancreas stained with hematoxylin and eosin from rats injected with dibutyltin dichloride (DBTC; 4-$\mu$m sections). (*A*) Normal pancreas (from naive and vehicle-injected animals) were similar in appearance. (*B*) Pancreas 3 days after DBTC injection in the tail vein (8 mg/kg body weight). The pancreas shows acinar atrophy (*arrowhead*), stromal proliferation (*thick arrow*), and edema in the interlobular and intralobular ducts (*thin arrow*). (*C*) Liver tissue section from a control Lewis rat stained with hematoxylin and eosin. (*D*) Liver section from a Lewis rat injected with DBTC taken 7 days after injection. No signs of inflammation are apparent in the liver. Scale bar = 100 mm.

single index of responsiveness to mechanical stimulation and morphine treatment. Injection of morphine sulfate had an antinociceptive effect on those animals that had developed pancreatitis and the behavioral changes described above. The antinociception produced was dose-dependent (fig. 5) but was highly significant with the 10-mg/kg dose of morphine. No changes in behavior of animals in control groups were evident for the three doses of morphine used.

### Immunohistochemical Studies

The same animals used for behavioral studies were killed for immunohistochemical procedures to minimize the total number of rats in these studies. Spinal cord tissue (T6–T10) was sampled from animals 3, 7, 14, and 21 days after DBTC injection and from controls. Figure 6A shows a control section for SP staining. The experimental pancreatitis group showed increased relative staining density for SP (fig. 6B). CGRP staining showed no apparent changes in density by qualitative visual inspection (figs. 6C and 6D). Quantitation of the staining

density showed that SP staining increased significantly on days 3 and 7 (fig. 7A). The CGRP staining did not increase significantly (fig. 7B).

## Discussion

The current study tested the hypothesis that the Lewis inbred strain of rats treated with DBTC developed serum and behavioral changes consistent with persistent pancreatitis.[8,12,13] Lewis rats expressing both behavioral and neurochemical changes exhibited clinical signs of pancreatitis during the first week (increased serum concentrations of amylase and lipase). Histologic signs of pancreatic inflammation were also observed during the first week. Inbred Lewis rats injected with DBTC developed increased cutaneous sensitivity demonstrated by an increase in withdrawals from von Frey fiber stimulation applied to the abdominal area as well as a decrease in thermal thresholds in the same region. Increased sensitivity in these measures may represent the "referred"

**A) von Frey 4.78 mN**



**B) von frey 9.96 mN**
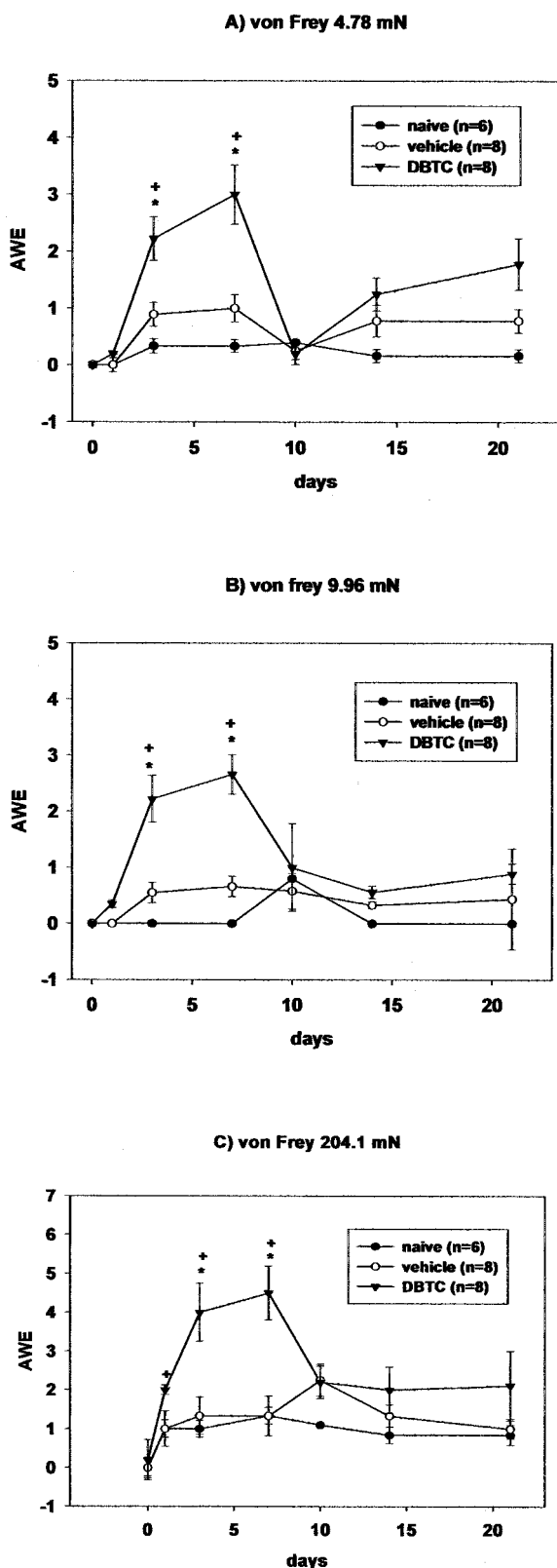


**C) von Frey 204.1 mN**



Fig. 3. Time course of nociceptive behaviors. The y-axis indicates the average number of reflexive withdrawals from von Frey stimuli applied to the abdominal surface of rats (AWE). (*A*) Rats with dibutyltin dichloride (DBTC) injection demonstrate a greater sensitivity to von Frey stimuli (4.86 mN) compared with naive and vehicle-injected animals at 3 and 7 days after DBTC injection. (*B*) Similar results were obtained with von Frey stimuli with an applied force of 9.96 mN. (*C*) Suprathreshold stimuli (von Frey 204.1 mN) also evoked greater reactivity from rats with DBTC injection compared with rats from the other groups. +$P$ < 0.05 when comparing the respective animal group behavior to its baseline measure at time point 0. *$P$ < 0.05 when compared with corresponding controls (naive and vehicle-injected groups). Error bars denote SEM.

ies where morphine attenuated similar pain-related indices.[7,36–38] The abdominal area from which stimulations evoked these behaviors was confined to zones documented in humans to be referred areas for pancreatic pain. This is supported by concurrent findings that the inflammation was restricted to the pancreas. This includes the presence of inflammatory cells in the histologic sections and methylene blue plasma extravasation. The histologic findings were normal for the liver, and methylene blue extravasation was not evident for the liver and abdominal wall. If the abdominal wall had been involved, a more widespread sensitization might have been present. Animals that demonstrated behavioral changes also expressed an increase in staining density for SP in the superficial laminae of the T6–T10 spinal cord sections sampled. This region receives the majority of primary afferent fibers that innervate the pancreas.[19,39]

Significant challenges have been encountered in attempts to develop animal models of pancreatic pain because of their acute nature and costly develop-
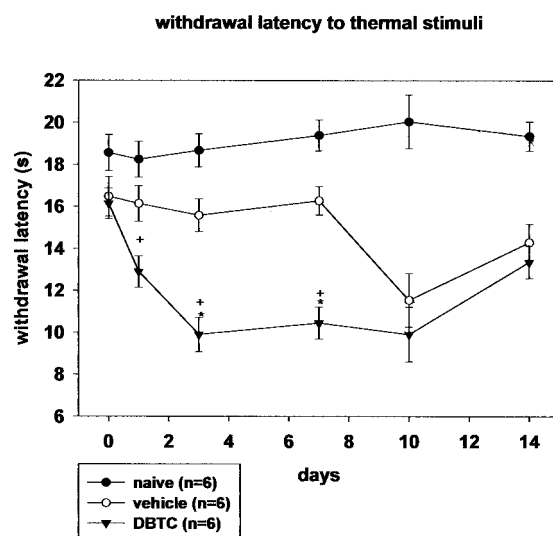
**withdrawal latency to thermal stimuli**



Fig. 4. Time course for the withdrawal latencies of rats injected with dibutyltin dichloride (DBTC), vehicle, or neither (naïve animals). Rats with DBTC injection demonstrated more rapid reflexive withdrawal to abdominal stimulation with a radiant heat source 3 and 7 days after injection compared with the vehicle-injected and naive groups. *$P$ < 0.05 compared with vehicle control. +$P$ < 0.05 compared with baseline (time point 0). Error bars denote SEM.

cutaneous pain experienced by patients with pancreatitis. These behavioral changes were reversed by morphine dose dependently, suggesting that these behaviors were nociceptive related, as demonstrated in other stud-
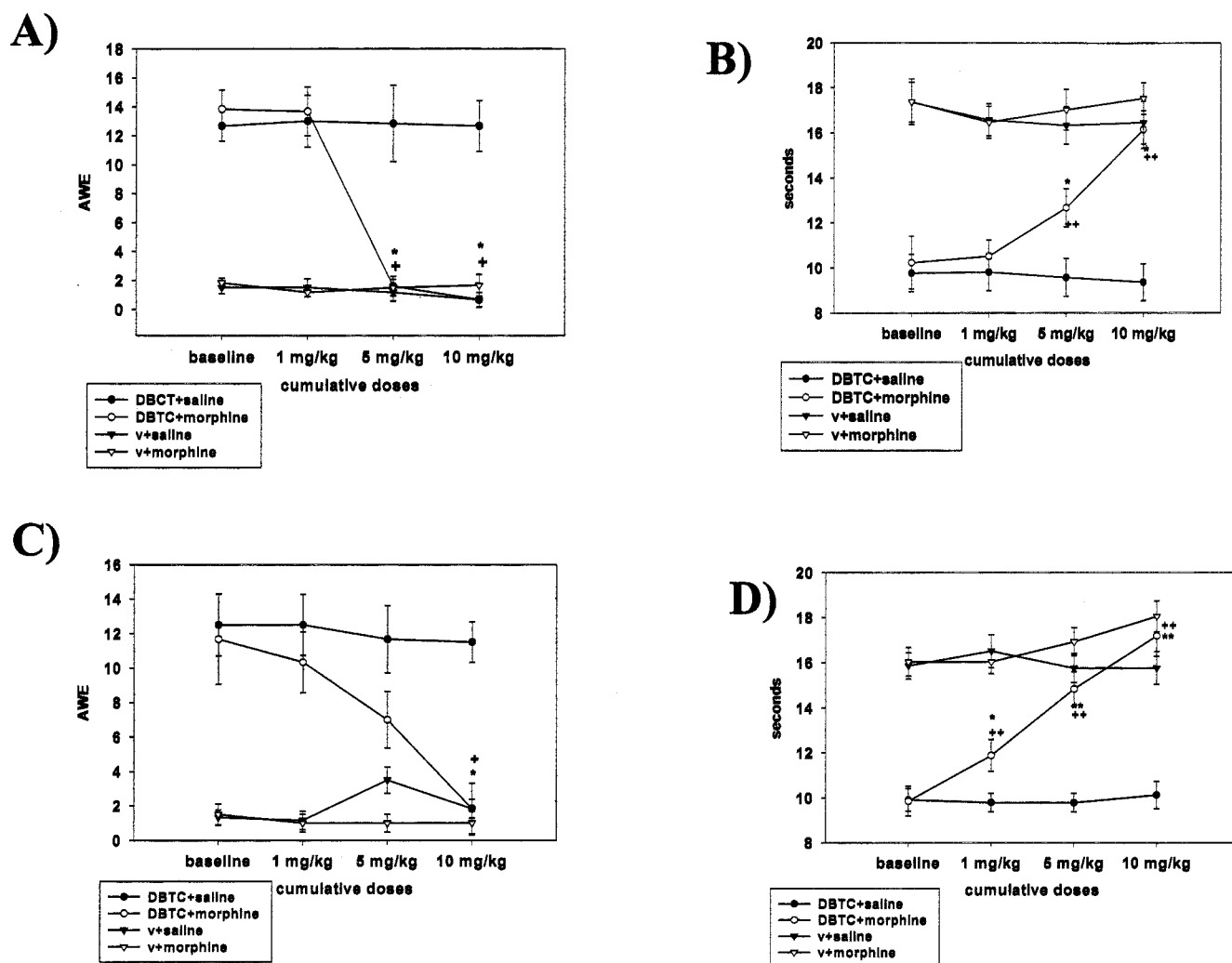
Fig. 5. Dose–response curves for nociceptive responses to von Frey stimuli (cumulative scores) and heat stimuli at days 3 and day 7 after either saline or morphine systemic injection in animals treated previously with either vehicle (v) injection or dibutyltin dichloride (DBTC) injection. *$P$ < 0.05 *versus* that group's own baseline (Student paired $t$ test). +$P$ < 0.05 comparing saline and morphine groups (Student unpaired $t$ test). **,++$P$ < 0.01. (*A*) Day 3 responses to von Frey stimuli. (*B*) Day 3 responses to heat stimuli. (*C*) Day 7 responses to von Frey stimuli. (*D*) Day 7 responses to heat stimuli. AWE = average withdrawal event (the summation of responses to three von Frey filaments; see Methods). Error bars denote SEM.

ment.[40-42] Some procedures creating pancreatitis are often invasive, introducing a number of extraneous experimental variables (abdominal surgery effects, behavioral inconsistencies, *etc.*).[43,44] The visceral pain described here is reproducible and useful for comparison of intragroup and intergroup relations and experimental conditions.[45] Many animal models of pancreatic pain use the induction of acute inflammation in the pancreas to establish changes in the periphery in the organ and dorsal root ganglia.[5,46] These inflammatory models are short-lived or lead to multiorgan failure, and thus their utility for study of persistent changes in visceral pain transmission at the level of the spinal cord are limited. The development of an animal model of persistent pancreatic inflammation with central changes revealed through behavioral testing will be useful for assessing the utility of pharmacologic agents that may reduce

visceral nociception. In the context of this animal model, persistent pancreatitis is considered an inflammatory episode that last more than 24–48 h, and thus it mimics one of the recurrent bouts of inflammation and abdominal pain experienced by patients with chronic pancreatitis, perhaps persisting because of ingestion of alcohol in our rats as in humans with chronic alcoholic pancreatitis. Clinical signs of pancreatitis in humans include increased serum concentrations of the pancreatic enzymes amylase and lipase plus a variety of other clinical signs.[14] If a pancreatic biopsy is performed in patients, histologic signs of inflammation are present in the pancreas, including invasion by inflammatory cells, acinar atrophy, ruptured or engorged pancreatic ducts, and interstitial edema.[47] These same histologic signs of inflammation and enzyme concentration changes were seen in the pancreas of rats injected with DBTC.
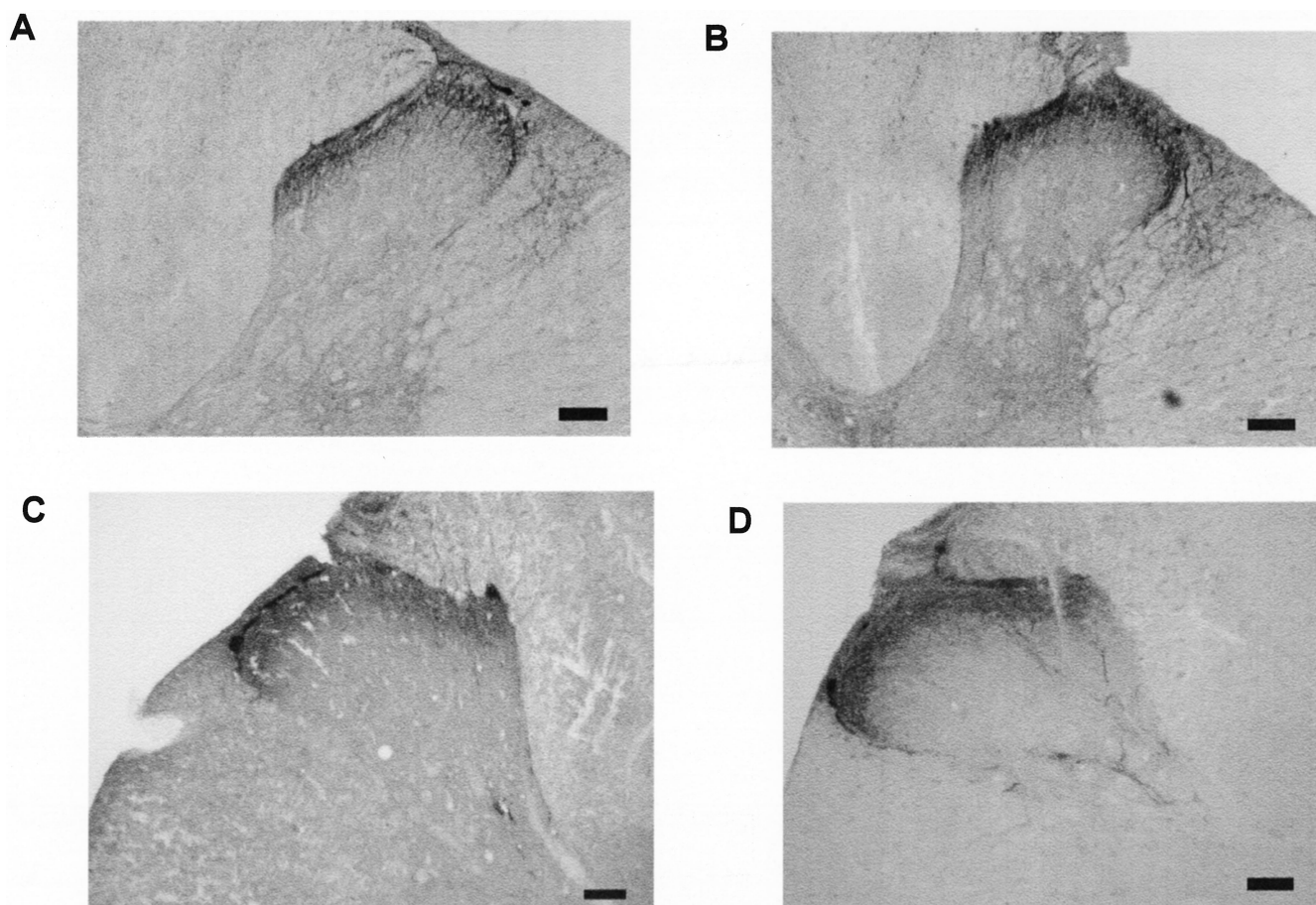
**Fig. 6. Representative photomicrographs of spinal cord sections showing staining for substance P (SP; *A* and *B*) and calcitonin gene-related peptide (CGRP; *C* and *D*) in the dorsal horn. (*A* and *C*) Sections taken from control animals; (*B* and *D*) sections taken from animals with pancreatic inflammation induced by tail vein injection of dibutyltin dichloride.**

Nociceptive behavioral reflex changes have been used in many studies as a reflection of the presence and extent of nociceptive changes in animals. Visceral pain is often "referred" to the abdominal dermatomes that receive their innervation from the same spinal segments that innervate the visceral organs.[11,12,48] Pain originating specifically from the pancreas is often "referred" to the upper abdomen and radiates to the back.[8,9] Measurements of nociceptive responses in a variety of experimental conditions have been used as valid quantitative methods to assess the equivalent of mechanical allodynia and thermal hyperalgesia in animal models.[1] In assessing visceral nociception, measurements of animal activity or pseudoaffective reflexes are often used.[7,45] Laird *et al.*[10,37] recently introduced the use of tests of cutaneous sensitivity to measure visceral nociception. In the current study we used a modified version of this method, quantifying a pseudoaffective reflex with supraspinal components (abdominal withdrawal and licking of the stimulated area after von Frey stimulation) as an index of evoked referred visceral pain. At the same time, thermal stimulation has been used to analyze the central sensitization induced by a variety of noxious manipulations.[49-51] In our study, thermal stimulation was used to test the cutaneous zones of referred nociceptive sensitivity with pancreatitis and to establish the presence of central sensitization in response to visceral inflammation. Our results suggest that increases in nociceptive responsivity persist only during the period of pancreatic inflammation demonstrated histologically and with serum enzymes (between 3 and 7 days; figs. 1 and 3). When the inflammation subsides, the nociceptive behaviors return to baseline. In the highly inbred strain of rats used by Merkord *et al.*, it is reported that the pancreatitis persists through 6 months.[31,52,53] The difference in duration of their model compared with the results of the current study is likely explained by their use of a highly inbred strain of rats, while we have attempted to repeat the results in the commercially available Lewis rat in which the pancreatitis and nociceptive behaviors persist for only the first 7–10 days. It is possible that the nociceptive behaviors we report here could also persist as chronic pancreatitic tissue becomes more fibrotic in the highly inbred model used by Merkord *et al.*[31]

While in humans with acute pancreatitis it is difficult to correlate inflammatory signs in the pancreas with

## Substance P Immunostaining



## CGRP Immunostaining
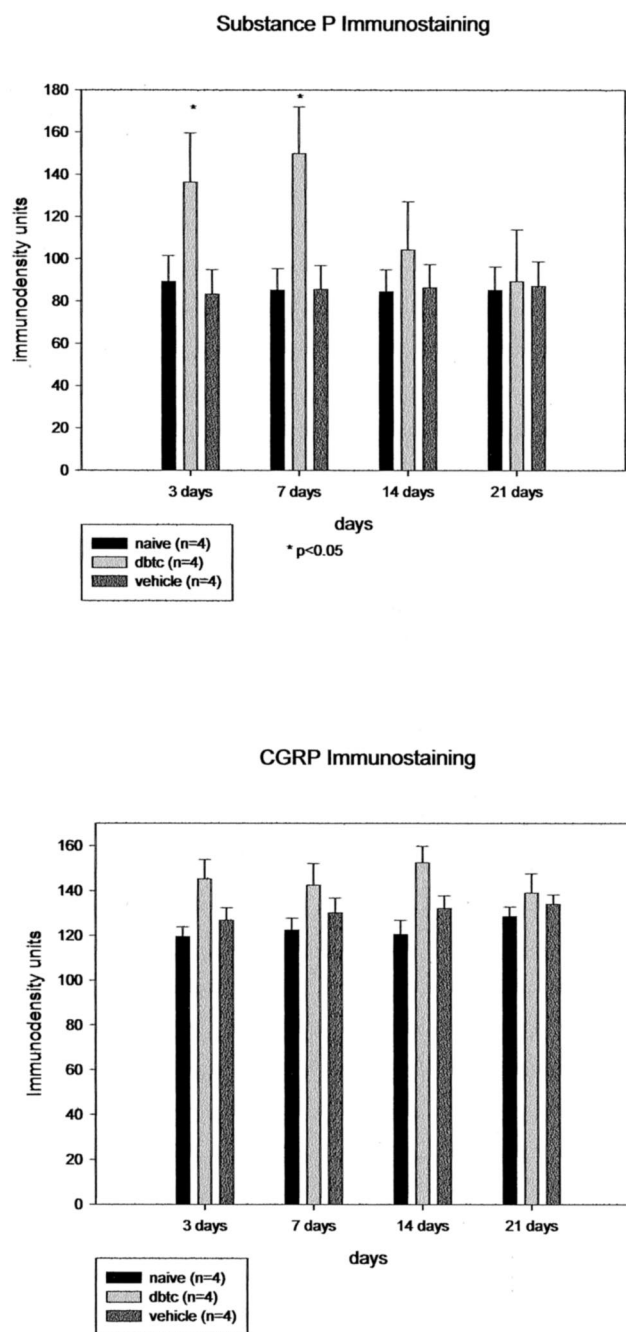


**Fig. 7. Time course for immunostaining density of substance P (SP) and calcitonin gene-related peptide (CGRP) in the superficial dorsal horn. (*A*) Quantitative immunohistochemistry demonstrates expression increases for SP at days 3 and 7 from animals injected with dibutyltin dichloride for comparison to the other groups (\*$P < 0.05$). (*B*) The CGRP immunostaining showed a trend of increased density at all time points, but the increase did not reach statistical significance. Error bars denote SEM.**

abdominal pain, in the case of animal models with chronic pancreatitis, pain and histopathologic changes can be correlated.[54] In our model, the correlation between inflammation and increased nociceptive responses observed suggest that this persistent pancreatitis produced in commercially available rats can be used

as a realistic model for this syndrome. These findings are consistent with previous studies reporting that inflammation of visceral tissue leads to states of increased pain.[2,55] During the course of chronic pancreatitis in humans, patients can suffer from several relapsing flareups of silent or mildly symptomatic acute pancreatitis that is accompanied by abdominal pain.[56] The 7–10-day time course of the nociceptive increases in this study suggests that this model can be useful in mimicking the time course of a pancreatic flare-up in the human condition.

The neuropeptide alterations were investigated in this study because most visceral afferent fibers innervating the pancreas contain SP and CGRP, and alterations in the spinal cord of these neuropeptides would be indicative of central nociceptive changes. These peptides are known to undergo alterations in their expression during inflammatory conditions in the viscera.[25,26,35,57,58] SP and CGRP have been shown to be involved in the maintenance of chronic pain conditions.[23,59] Antagonists of the SP receptor (NK-1) can abrogate visceral pain responses.[60,61] The NK-1 receptor is involved in the generation and maintenance of hyperalgesia,[62,63] and its expression has been correlated with chronic pain states, including pain in chronic pancreatitis.[64] CGRP also plays a significant role in visceral nociception, and a synergistic action between SP and CGRP has been suggested based on the potentiation of SP-induced responses by CGRP.[65-68] In our study, increased expression of SP immunoreactivity is observed at the same time points at which the animals with induced pancreatitis express nociceptive behaviors. The immunoreactivity for CGRP showed a trend to increase, but the increase did not reach statistical significance. These results suggest the involvement of SP in the maintenance of the pain state that develops after inflammation of the pancreas, and likely these central changes also contribute to the referred cutaneous nociceptive responses. A role for CGRP cannot be ruled out, however, in light of the ability of CGRP to maintain large intracellular stores and a different time course of dynamic changes.[23,69] The increased expression of SP is perhaps an indication of peripheral nerve and central changes occurring in responses to increased afferent barrage from the inflamed pancreas and the resultant increased release of SP in the spinal cord.[27] At the same time, it is possible that the origin of increased expression of SP might not only be visceral afferents as the animals used for the immunohistochemical analysis also demonstrated increased sensitivity to somatic stimulation (mechanical von Frey and thermal stimulation). It has been demonstrated previously that short-term noxious stimulation can induce SP release in the spinal cord.[70] The persistent animal model described here has as an advantage, characteristics of longer-term inflammation, including indications of a sensitized state in the spinal cord that could lead to long-term changes.[6]

Sensitization of these animals to a second pancreatic insult or other noxious manipulations has not yet been attempted and would require further studies.

In conclusion, an animal model of persistent pancreatitis with a 1-week time course has been developed in commercially available rats that demonstrate nociceptive behavioral responses. These behaviors are responsive to morphine, which suggests that these behaviors are nociceptive-specific. Neuropeptide alterations in the spinal cord and the presence of altered nociceptive responses in referred cutaneous sites indicate an alteration of nociceptive processing in the central nervous system. These studies provide insight into the neurochemistry of this disease process at the spinal cord level and form the basis for further characterization and validation of this model for investigation of visceral nociceptive processing.

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