Spinal Microglia Initiate and Maintain Hyperalgesia in a Rat Model of Chronic Pancreatitis

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**Background & Aims:** The chronic, persistent pain associated with chronic pancreatitis (CP) has many characteristics of neuropathic pain, initiated and maintained by the activation of spinal microglia. We investigated whether activated microglia in the thoracic spinal cord contribute to chronic pain in a rat model of CP. **Methods:** CP was induced in Sprague–Dawley rats by an intraductal injection of 2% trinitrobenzene sulfonic acid (TNBS). Hyperalgesia was assessed by the measurement of mechanical sensitivity of the abdomen and nociceptive behavior to electrical stimulation of the pancreas. Three weeks after induction of CP, spinal samples were analyzed by immunostaining and immunoblot analyses for levels of CD11 (a marker of microglia, determined with the antibody OX42) and phosphorylated p38 (P-p38, a marker of activation of p38 mitogen-activated protein kinase signaling). We examined the effects of minocycline (inhibitor of microglia) and fractalkine (microglia-activating factor) on visceral hyperalgesia in rats with CP. **Results:** Rats with CP had increased sensitivity and nociceptive behaviors to mechanical probing of the abdomen and electrical stimulation of the pancreas. The dorsal horn of the thoracic spinal cords of rats with CP contained activated microglia (based on increased staining with OX42), with an ameboid appearance. Levels of P-p38 increased in rats with CP and colocalized with OX42-positive cells. Intrathecal injection of minocycline reversed and prevented the increase of nocifensive behavior and levels of P-p38 in rats with CP. Fractalkine induced hyperalgesia in rats without CP, which was blocked by minocycline. **Conclusions:** Activated spinal microglia have important roles in maintaining and initiating chronic pain in a rat model of CP. Microglia might be a target for treatment of hyperalgesia caused by pancreatic inflammation.

**Keywords:** Central Nervous System; Macrophage; Sensory Neuron; Microglial Activation.

Chronic pancreatitis (CP) is a disorder of persistent inflammation, fibrosis, and destruction of the glandular pancreas most often caused by excessive alcohol ingestion. Chronic epigastric pain, the cardinal feature of CP, is usually recurrent, intense, and long-lasting. The pain in CP can lead to malnutrition, narcotic addiction, and major socioeconomic problems. The control of the pain in CP remains difficult and frustrating. The origin of pain in CP is supposed to be from pancreatic (increased pressure in pancreatic tissue, ischemia, fibrosis, pseudocyst formation, inflammation, or an alteration of pancreatic nerves) and extrapancreatic (duodenal or bile duct stenosis or maldigestion) causes. However, the pathogenesis of pain in CP remains to be elucidated. Recently, it has been noted that the pain in CP shares many characteristics of neuropathic pain. For example, an up-regulation of substance P and calcitonin gene-related peptide has been observed in the dorsal root ganglia and spinal dorsal horn in the thoracic segment. The molecular/cellular mechanisms of the pain in CP have also emerged in recent years.

Microglia, the intrinsic macrophages of the central nervous system, are activated in the spinal cord in neuropathic or inflammatory pain. For neuropathic pain, activated microglia is found to have a causal role of pain hypersensitivity following nerve injury, including ligation of the sciatic nerve, spinal nerves, or lumbar spinal cord roots; spinal cord injury; or spinal infection with the human immunodeficiency virus. For inflammatory pain, spinal microglial activation is shown following intra-articular injection of complete Freund’s adjuvant. Microglia also contribute to the development of visceral hyperalgesia in the colon either with or without inflammation. An animal CP model using an instillation of trinitrobenzene sulfonic acid (TNBS) into the biliopancreatic ducts of rats has been developed that displays behavior and morphologic changes similar to those seen in humans with CP. Using this CP model, we tested the hypothesis that spinal microglial activation...
mediates the maintenance and initiation of chronic visceral pain in CP.

Materials and Methods

Animals

Adult male Sprague–Dawley rats (300–400 g) were used (Laboratory Animal Center, National Yang-Ming University, Taipei, Taiwan). The rats were housed at a temperature of 23 ± 3°C and maintained on a 12-hour light-dark cycle (lights on at 7 AM) with standard laboratory chow and tap water available ad libitum. Experiments were performed during the light cycle. All protocols were approved by the Institutional Animal Care Committee at National Yang-Ming University.

Induction of CP and Electrode Implantation

Rats were anesthetized with sodium pentobarbital (45 mg/kg intraperitoneally), and CP was induced as described previously. Briefly, the common bile duct was closed temporarily near the liver with a small vascular clamp. A blunt 28-gauge needle with PE10 tubing attached was inserted into the duodenum and was guided through the papilla into the duodenum and secured with sutures. A total of 0.5 mL of a 2% solution of TNBS (Sigma, St Louis, MO), in 10% ethanol in phosphate-buffered saline (PBS), or vehicle was infused into the pancreatic duct over 2 to 5 minutes at a pressure of 50 mm Hg. After a 30-minute exposure to TNBS, the needle and tubing were removed, the hole in the duodenum was sutured, and the vascular clamp was removed to restore the bile flow. In some groups of rats, a pair of electrodes was attached to the pancreas and externalized behind the head after the infusion of the pancreatic duct with vehicle or TNBS. Animals were returned to their housing and given free access to drinking water and standard food pellets.

Surgical Implantation of an Intrathecal Catheter

The surgical implantation of intrathecal (IT) catheters was performed together with the induction of CP. A 7-cm PE10 tube connected to a 4-cm PE50 tube was inserted through a small puncture in the atlanto-occipital membrane of the cisterna magna so that the caudal tip reached the T10 level of the spinal cord. The catheters were primed with vehicle before implantation, and the neck incision was closed with sutures. The catheter was left in place until the required experiments were completed. The catheter position was verified in each animal by a postmortem examination of the spinal cord at the end of the experiment.

Measurement of Hyperalgesia in Rats With CP

Von Frey filament test. Von Frey filament (VFF) test was performed as described previously. Briefly, before testing, the belly was shaved and areas designated for stimulation were marked. Rats were placed in a plastic cage with a mesh floor and were given 30 minutes to adapt before testing. VFFs of 6 different calibers (0.4, 0.6, 1.4, 4, 6, 10 g; North Coast Medical, Morgan Hill, CA) were applied to the abdomen in ascending order 10 times, each for 1 to 2 seconds with a 10-second interval between applications. A positive response consisted of the rat raising its belly (withdrawal response). All of the tests were performed in a blinded manner.

Electrical stimulation of the pancreas. In the rats prepared with electrodes attached to the pancreas, 3 weeks after infusion of TNBS/vehicle, successive applications of current at 2, 5, and 10 mA for 5 minutes with a 10-minute rest between stimulation periods were administered to the rats. The numbers of nociceptive behaviors observed during the 5 minutes of the electrical stimulation period were blindly counted by an assistant. The nociceptive behaviors consisted of stretching, licking of the limbs and abdomen, contraction of abdominal wall muscles, and an extension of the hind limbs as described previously in a CP model.

Histologic Examination of the Pancreas and Immunohistochemical Staining of the Spinal Cord

After the required experiments, the rats were killed under anesthesia with pentobarbital (100 mg/kg intraperitoneally) and transcardially perfused with 300 mL normal saline followed by 500 mL 4% paraformaldehyde in ice-cold PBS. The pancreas and spinal cord (T1–S2) were removed and postfixed for 4 hours in 4% paraformaldehyde and cryoprotected overnight in 30% sucrose in PBS. The pancreas was embedded in OCT, cut into 10-µm frozen sections, stained with H&E, and observed under a light microscope. The severity of pancreatitis was evaluated based on the appearance of segmental gland atrophy, periductular and intralobular fibrosis, and the presence of acute and chronic inflammatory infiltrates.

The methods for immunohistochemistry and for quantification of spinal microglial activation are shown in Supplementary Methods and Materials.

Western Blots

For Western blot analysis, groups of rats with or without TNBS-induced CP were used. After the rats were deeply anesthetized with pentobarbital (100 mg/kg intraperitoneally), the T8 to T12 levels of the spinal cord were quickly removed and placed in a dish with ice-cold PBS. The spinal cords were homogenized in a tissue protein extraction reagent (Thermo Scientific, Rockford, IL) that contained protease inhibitors and the phosphatase inhibitor cocktail 1/2 (Sigma). The resulting homogenate was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Millipore Corp, Bedford, MA) or an anti-p38 antibody (1:1000; Cell Signaling Technology, Boston, MA) or an anti-p38 antibody (1:1000; Cell Signaling Technology) in PBS containing 1% bovine serum albumin overnight at 4°C. After washing in PBS with 0.5% Tween 20, the membranes were incubated with an anti-phosphorylated p38 (P-p38) antibody (1:1000; Cell Signaling Technology, Boston, MA) or an anti-p38 antibody (1:1000; Cell Signaling Technology) in PBS containing 1% bovine serum albumin overnight at 4°C. After washing in PBS with 0.5% Tween 20, the membranes were incubated with a horseradish peroxidase–conjugated secondary antibody (1:5000; Jackson ImmunoResearch, West Grove, PA) in PBS containing 1% bovine serum albumin for 2 hours at room temperature. The bands were visualized using the Western Lighting Chemiluminescence Reagent Plus Kit (PerkinElmer, Waltham, MA) and appropriate exposure to Kodak X-ray film (Kodak, Rochester, NY). Membranes were subsequently stripped and reprobed for actin (anti-β actin, 1:10,000; Abfrontier, Seoul, Korea). Films were scanned, and band intensities were measured using a Luminescence Imaging System (FujiFilm LAS-4000; FUJIFILM, Tokyo, Japan). Quantification was performed using Multi-Gauge software (version 3.0, FUJIFILM).
**Experimental Design**

**Experiment 1: validation of a rat model of CP with hyperalgesia.** The purpose of this experiment was to validate an animal model of CP with chronic hyperalgesia. We followed the protocol designed by Pasricha’s group. Briefly, TNBS or vehicle was injected into the pancreatic duct of Sprague–Dawley rats. Three weeks after the TNBS/vehicle injection, nociception was assessed either by measuring the mechanical sensitivity of the abdomen (n = 5 in each TNBS or vehicle group, VFF test) or by recording the number of nocifensive behaviors in response to electrical stimulation of the pancreas (n = 8 in each group). The pancreas was removed from rats for the evaluation of the existence of CP using H&E stain in the group testing for VFF.

**Experiment 2: evidence of spinal microglial activation after CP: immunohistochemistry and Western blotting.** At 1 day, 1 week, and 3 weeks after the TNBS or vehicle intrapancreatic duct injection, the rats were killed and samples from the spinal cord were collected. The spinal cord was processed for immunohistochemistry for OX42. Parts of the sample sections (T8–T12) were double labeled for P-p38/OX42, P-p38/GFAP, or P-p38/NeuN. For Western blots, another 2 groups (n = 4 in each group) with similar treatments were killed, and spinal samples of the thoracic spinal cord were collected and processed for Western blotting for P-p38 and p38.

**Experiment 3: the role of microglia in the maintenance of CP-related hyperalgesia: the effect of minocycline.** After the establishment of CP and its related hyperalgesia, minocycline (100 μg/10 μL IT; Sigma) or vehicle was given every 12 hours from the afternoon of the 21st day to the morning of the 28th day (14 doses) after the TNBS or vehicle injection. The mechanical sensitivity of the abdomen was assessed at the 28th day in 4 groups of rats (TNBS-vehicle [T + V], TNBS-minocycline [T + M], vehicle-vehicle [V + V], and vehicle-minocycline [V + M], n = 5–6 in each group) with VFF test. After VFF test, the rats were killed, and samples of the thoracic spinal cord were collected and processed for Western blotting for P-p38/p38. Nociceptive behaviors elicited by electrical stimulation were also evaluated in another 4 similar groups (n = 4–5 in each group) of rats. Then, the 4 groups (n = 4–5 in each group) of rats were assessed with VFF or electrical stimulation as described previously.

**Statistical Analysis**

Data are presented as mean ± SEM. The intensities of OX42 staining (microglia activation response) were compared using independent-samples t test (between vehicle- and TNBS-treated groups) or one-way analysis of variance (ANOVA) post hoc by Tukey test (among 3 time points). Behavioral data from VFF and electrical stimulation were analyzed by 2-way mixed-design ANOVA and post hoc by independent-samples t test or Tukey test. For Western blots, data were analyzed by independent-samples t test or one-way ANOVA and post hoc by Tukey test. To correlate the nociceptive behavior to von Frey stimulus with the spinal P-p38 expression, Pearson’s r correlation was used. For this analysis, percentage of change in P-p38 measured from Western blots was correlated with response frequency to von Frey stimulus. Differences were considered statistically significant at P < .05.

**Results**

**Experiment 1: Validation of a Rat Model for CP With Hyperalgesia**

The sensitivity of TNBS- and saline-treated rats in response to VFF of various strengths from 0.4 to 10 g 3 weeks after TNBS/vehicle infusion into the pancreatic duct was examined. The response frequencies to VFF at 3 weeks in TNBS-treated rats were significantly higher in a dose-responsive manner than those in saline-treated rats (P = .001). Therefore, we confirmed that the pancreatic infusion of TNBS produced a marked increase in the sensitivity to mechanical probing of the abdomen (Figure 1A). The numbers of nocifensive behaviors on electrical stimulation directly to pancreas also significantly increased in a dose-responsive manner in TNBS-treated rats compared with controls (P < .001) (Figure 1B). Histologic examination showed normal pancreas in vehicle-treated rats (Figure 1C), but dense inflammatory infiltrates, large regions of acinar loss, and fibrosis replaced the normal pancreas in TNBS-treated rats (Figure 1D).

**Experiment 2: Evidence of Spinal Microglia Activation After CP: Quantitative Immunohistochemistry and Western Blotting**

Three weeks after TNBS or vehicle treatment, immunohistochemistry revealed an increase in OX42 staining in the spinal dorsal horn from rats with CP compared with controls. Furthermore, in the vehicle-treated rats, antibodies against OX42 showed that the resident microglia exhibited morphology of the quiescent stage (ie, small compact somata bearing long, thin, ramified processes; Figure 2A), but microglia in the TNBS-treated rats exhibited an activated phenotype (ie, marked cellular hypertrophy and retraction of cytoplasmic processes; Figure 2B). This finding suggested an activation of microglia in the
thoracic spinal cord of rats with CP. The increase was mainly located at the spinal level receiving direct afferent input from the pancreas (T8–T12) but could also be observed in the neighboring thoracic and lumbar segments (Supplementary Figure 1). The spinal microglia activation (increased OX42 expression) occurred at the first day after the induction of pancreatitis, progressively increased at day 7, and reached its maximum at 3 weeks (Supplementary Figure 2).

Because prior studies have shown that functional microglial activation is associated with the phosphorylation of p38,13 we performed immunohistochemistry for spinal P-p38 in the rats with CP. As shown in Figure 2C to E, the immunoreactivity for P-p38 was colocalized with OX42-positive cells (Figure 2E) but not with NeuN-positive cells (neuronal marker) (Figure 2C) or with GFAP-positive cells (astrocyte marker) (Figure 2D). We used Western blots to quantify P-p38 levels in the thoracic spinal cord in both TNBS- and vehicle-treated groups. Increased levels of P-p38 were noted in rats with CP compared with controls ($P < .05$), but total p38 levels from the vehicle- and TNBS-treated groups were similar (Figure 3A and B). These findings suggested that CP-induced spinal microglia activation is associated with activation of p38.

**Experiment 3: Effect of Minocycline on the Treatment of CP-Related Hyperalgesia**

Once the CP-related hyperalgesia was established, IT minocycline treatment reversed the hyperalgesia on mechanical and electrical stimulation in rats with TNBS-induced CP back to control levels ($P = .001; $ Figure 4A and B). As shown in Figure 4C, the expression of P-p38 proteins was significantly increased in the TNBS-treated group, and this increase was reversed by the treatment of IT minocycline ($P = .009$). Total p38 was similar across the 4 groups (Figure 4D). The visceral hyperalgesia to mechanical stimulation reappeared once minocycline was stopped (Figure 4E).

**Experiment 4: Effect of Minocycline on the Prevention of CP-Related Hyperalgesia**

In the rats pretreated with IT minocycline 30 minutes before the infusion of TNBS, no significant differences were noted in the nociceptive responses to either the VFF test or electrical stimulation compared with the vehicle-treated groups (Figure 5A and B). In the rats pretreated with vehicle before the infusion of TNBS, the TNBS-induced hyperalgesia was preserved, as evidenced by the increased response frequencies to VFF ($P < .001; $ Figure 5A) and the higher numbers of nocifensive behaviors on electrical stimulation to the pancreas ($P < .001; $ Figure 5B). Western blot analysis also showed that the increased spinal expression of P-p38 in the rats with CP was completely blocked by pretreatment with IT minocycline ($P = .01; $ Figure 5C). The total p38 levels were similar across the 4 groups (Figure 5D). These data suggested that activated spinal microglia may mediate the initiation of
chronic pain in CP, possibly via the p38–mitogen-activated protein kinase (MAPK) pathway.

By pooling data from the rats that underwent both P-p38 measurement and behavioral assays in experiments 2 to 4, there was a significant correlation between percentage changes of P-p38 and nociceptive behavior ($r = 0.673$, $P < .001$) (Supplementary Figure 3).

**Experiment 5: Effect of the Microglial Activating Factor Fractalkine on Hypersensitivity in Non-CP Rats**

To further corroborate the involvement of microglial activation in the development of CP-related hypersensitivity, we evaluated the effect of fractalkine on hyperalgesia to mechanical and electrical stimulation. In the vehicle-treated rats, the response frequency to VFF ($P = .01$; Figure 6A) and the numbers of nociceptive behaviors on electrical stimulation to the pancreas ($P < .001$; Figure 6B) were significantly increased after an IT injection of fractalkine. In contrast, in the rats pretreated with minocycline (100 µg/rat in 10 µL IT) followed by an IT injection of fractalkine, the enhanced nociceptive responses were reversed to baseline (Figure 6A and B).

**Discussion**

Using a validated CP rat model, we showed that there was a large shift in microglial status from a resting state to an activated state in the thoracic spinal dorsal horn 3 weeks after induction of CP. Our results suggested that chronic visceral inflammation might result in prolonged spinal microglial activation, which supports a critical role of spinal microglia in the maintenance of pain in CP.

![Figure 2](image1.png)

**Figure 2.** Immunohistochemistry of spinal microglia. (A) In vehicle-treated rats, the morphology of spinal microglia displayed features of the resting state showing small, compact somata bearing long, thin, and ramified processes. (B) Three weeks after induction of CP, spinal microglia exhibited the activated phenotype showing cellular hypertrophy and retraction of processes. The density of spinal OX42 is also significantly greater in TNBS-treated than in vehicle-treated rats. In the thoracic spinal samples from TNBS-treated rats, P-p38 (red) staining was not colocalized with (C) GFAP-positive cells (green) of (D) NeuN-positive cells (green) but with (E) OX42-positive cells (green). Bar = 100 µm in panels A and B and 25 µm in panels C–E. Arrows indicate the microglia colocalized with P-p38.

![Figure 3](image2.png)

**Figure 3.** Western blot analysis of the mean densitometry levels of spinal P-p38 and total p38 levels 3 weeks after TNBS or vehicle treatment. (A) P-p38 expression was significantly increased in TNBS-treated compared with vehicle-treated rats. *$P < .05$, independent-samples t test. (B) Total p38 level was similar between the 2 groups. Upper panels are the representative Western blots showing levels of P-p38, p38, and β-actin in the thoracic spinal cord.
We also showed that activated spinal microglia may contribute to the maintenance of chronic visceral pain in CP by pharmacologically down-regulating microglial activity with the inhibitor minocycline. We showed that minocycline reversed nociceptive behaviors 3 weeks after an intraductal injection of TNBS. However, after the withdrawal of minocycline, the nociceptive behaviors returned to baseline. Our results echo the minocycline treatment effect in previous studies involving chronic neuropathic pain or visceral pain. In a chronic pain model induced by spinal cord injury (4 weeks after T9 spinal cord contusion injury), IT minocycline (100 μg/5 μL) rapidly attenuates the hyperresponsiveness of lumbar dorsal horn neurons and restores the nociceptive threshold of pain behaviors. Immediately after cessation of minocycline, mechanical thresholds returned to predrug levels and were equivalent to that of untreated animals, which had results that were similar to our findings. Our results are also in line with another visceral pain study showing that a single intraperitoneal injection of minocycline (6 mg/mL) reverses the chronic hypersensitivity in adulthood, induced by repeated colorectal distentions during the neonatal period. By contrast, systemic minocycline showed no effect.

Figure 4. Effect of IT minocycline (100 μg) treatment on the maintenance of hyperalgesia in the CP rat model. (A) The abdominal reflex on VFF test in rats after TNBS and vehicle treatment (T + V) was significantly increased compared with that in the control rats (V + V), in which hyperalgesia was reversed by an IT injection of minocycline (T + M). (B) The numbers of nocifensive behaviors on electrical stimulation of the pancreas were significantly higher in TNBS-treated rats (T + V) than those in the control rats (V + V), in which hyperalgesia was reversed by IT injection of minocycline (T + M) (P = .001, mixed-design 2-way ANOVA). (C) Significantly higher P-p38 levels were observed in the TNBS-treated rats (T + V) than that in the control rats (V + V), while IT injection of minocycline reversed the increased P-p38 levels (M + T) (P = .009, one-way ANOVA); *P < .01 T + V vs V + V or V + M; †P < .05 T + M vs T + V, one-way ANOVA followed by the Tukey post hoc test. (D) There were no differences in total p38 levels among the 4 groups (P = .87, one-way ANOVA). (E) Following 7 days of IT minocycline treatment (21st to 28th day), the mechanical hyperalgesia to a 10 g VFF filament stimulation was significantly decreased. The mechanical reflex returned to predrug level after stopping minocycline. *P < .05, independent-samples t-test. B, baseline.
with acute colon distention. Furthermore, IT minocycline (100–250 μg) would partially reverse existing bilateral mechanical allodynia induced by zymosan-associated sciatic inflammatory neuropathy but is not effective in reversing the established allodynia if the drug is given 1 week after the onset.19 In an L5 spinal nerve transection model, intraperitoneal injection of minocycline (10–40 mg/kg) 5 days after transection did not attenuate allodynia and hyperalgesia.20

In a visceral hypersensitivity rat model induced by daily water avoidance (1 hour per day for 10 consecutive days), concomitant administration of continuous IT infusion of minocycline during water avoidance (120 μg/day, from day 1 to day 10) would block the water avoidance–induced visceral hyperalgesia in adult rats at the 11th day.18 Despite the finding, it remained unclear whether minocycline (treatment after the 11th day) would be effective in treating established water avoidance–induced visceral hyperalgesia. Taken together, the previously described results may suggest that minocycline treatment in existing acute or chronic pain could be dependent on the site of injection, the dosage of minocycline, the type of injury, and the timing of treatment. Compared with previous studies, our treatment protocol is more rigorous and logical as a rationale for the efficacy of minocycline in studies of chronic visceral inflammation.

Another novel finding of the current study is that pretreatment with minocycline during the period of the intraductal injection of TNBS prevented the occurrence of chronic visceral hyperalgesia 3 weeks after induction of CP. Although pretreatment with minocycline has been repeatedly shown to prevent nociceptive behaviors in somatic pain models, such as chronic constriction injury,21,22 the formalin/carrageenan inflammatory model,23 IT N-methyl-D-aspartate–induced hyperalgesia,23 and sciatic inflammatory neuropathy,19 no previous study has shown that pretreatment with minocycline can prevent the development of visceral pain/hyperalgesia. Furthermore, the duration of the prevention of nociception after pretreatment with minocycline in these somatic pain models is usually short, ranging from hours to days.23 In a rat model of chronic constriction injury, pretreatment with minocycline delays the development of mechanical hyperalgesia until day 14.22 The most striking findings of the current study were that the long-term administration of minocycline, which began during the induction of CP, delayed the development of hypersensitivity for at least 3 weeks. We first showed that the nociceptive behaviors could be prevented up to 3 weeks after induction of CP. This phenomenon is also associated with the inhibition of spinal microglia. Because minocycline is devoid of any effect on neurons and astrocytes,24 it seems likely that the attenuation of hypersensitivity could be due to the selective inhibition of microglial activation. Whether the pre-

Figure 5. Effect of pretreatment with IT minocycline (100 μg) on the prevention of hyperalgesia in the CP rat model. (A) The mean abdominal reflexes to VFF test in TNBS-treated rats (V + T) were significantly higher than in the control rats (V + V), in which effect was prevented by an IT injection of minocycline (M + T) (P < .001, mixed-design 2-way ANOVA). (B) The numbers of nociceptive behaviors on electrical stimulation of the pancreas in TNBS-treated rats (V + T) were significantly increased compared with the control rats (V + V), in which effect was prevented by pretreatment with IT minocycline (M + T) (P = .01, one-way ANOVA). (C) Higher levels of P-p38 were observed in the TNBS-treated group (V + T) compared with the vehicle-treated group (V + V). This P-p38 level was blocked by pretreatment with IT minocycline (M + T) (P = .01, mixed-design 2-way ANOVA). (D) There was no difference in total p38 levels among the 4 groups (P = .77, one-way ANOVA).

\*P < .01 V + T vs V + V or M + V; \#P < .05 M + T vs V + T, one-way ANOVA followed by the Tukey post hoc test.
vvention effect of minocycline can be clinically useful to
patients with acute pancreatitis deserves further study.
P38, a member of the MAPK family, is activated in
spinal microglia after spinal nerve injury and is implicated
in producing neuropathic pain. A large body of liter-
ature also documents that p38 MAPK inhibition reduces
alldynia and hyperalgesia in several models of inflam-
matory and neuropathic pain, which suggests an important
role of this signaling pathway in somatic pain processing.
In the visceral hypersensitivity model induced by 10 days
of water avoidance, spinal P-p38 level is significantly in-
creased at day 11 and is blocked by IT minocycline. We
also showed a significant increase in spinal P-p38 levels 3
weeks after induction of CP that was reversed blocked by
IT minocycline and paralleled nociceptive behaviors and
spinal microglial activation. Our results suggested that
both the maintenance and initiation of chronic visceral
pain in CP relied on the p38 MAPK pathway for spinal
microglial activation. Despite this fact, the mechanism by
which P38 MAPK activation occurred in our CP model
and the downstream targets by which p38 may contribute
to spinal sensitization are not well elucidated. The poten-
tial downstream pathway of p38 phosphorylation may
involve the activation of transcription factors (such as
protein kinases) and the activation or enhancement of the
activity of other substrates (such as calcium-dependent
phospholipase A), which have been reported in associa-
tion with the facilitation of pain-related signaling.25

Fractalkine has been administered to rodents in many
studies to influence chronic pain behavior, mainly in
somatic pain. Fractalkine binds to its specific receptor,
CX3CR-1, which is expressed predominantly by spinal
cord microglia, to cause microglial activation.26–29 There-
fore, fractalkine is often considered to be a specific neu-
ron-to-microglia signal. According to several studies using
somatosensory behavioral tests, the IT administration of
fractalkine evokes dose-dependent mechanical allodynia
and thermal hyperalgesia in naïve rats.30 In the spinal
cord, concomitant with microglial activation, CX3CR-1
expression is up-regulated after chronic constriction in-
jury of the sciatic nerve.31 Recently, fractalkine adminis-
tration has been shown to increase abdominal electro-
myographic responses to noxious colorectal distention in
control rats, and this hypersensitivity can further be pre-
vented by IT minocycline treatment.16,18 In the current
study, we not only confirmed that fractalkine enhanced
the abdominal reflex of the VFF test, but it also increased
nocifensive behaviors of electrical stimulation directly to
pancreatic tissue. Both behaviors were further blocked by
IT minocycline. Taken together, these data support the
concept that the transmission of visceral nociceptive sig-
als may be enhanced through spinal microglial activa-
tion. However, the response to fractalkine in naïve rats
was not as robust as that to rats with CP. This phenom-
enon may be due to the fact that the fractalkine-associ-
ated CX3CR-1 pathway is not the only player in spinal
microglia activation. The microglial recruitment also de-
PENDS on signaling pathways involving TLR2 and TLR4
and on the chemokine CCL2 acting on CCR2. Other
factors in addition to microglia activation may also be
involved in the pathogenesis of persistent pain in CP.

In conclusion, we have provided novel evidence that
spinal microglial activation may play an important role in
the initiation and maintenance of chronic pain in CP. We
observed an increase in nociceptive behaviors and immu-
noreactivity for P-p38 in spinal cord sections from rats
with CP. P-p38 was colocalized with the microglial marker
OX42, which suggests that the activated microglia paral-
leled the activation of the p38 signaling pathway. Min-
ocycline, a microglial inhibitor, reversed or inhibited CP-
related hyperalgesia, possibly through a P-p38 pathway.
These findings open new perspectives in the understand-
ing of the mechanisms underlying chronic pain associated with CP in rats and may provide a chance for new drug development by targeting spinal microglia in humans.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.09.041.

References

Supplementary Materials and Methods

Immunohistochemistry

For immunohistochemistry of the spinal cord, transverse free-floating spinal sections (30 μm) were cut on a cryostat and processed for immunostaining with immunofluorescence. All of the sections were blocked with 2% donkey serum in 0.3% Triton X-100 for 1 hour at room temperature and incubated over 2 nights at 4°C with an antibody to OX42 (CD11b, a microglial marker; 1:200; Santa Cruz Biotechnology, Heidelberg, Germany). The sections were then incubated for 1 hour at room temperature with a Cy3-conjugated secondary antibody (1:800; Jackson ImmunoResearch, West Grove, PA). For double immunofluorescence, spinal sections were incubated with a mixture of polyclonal P-p38 and monoclonal neuronal-specific nuclear protein (NeuN) (a neuronal marker; 1:500; Chemicon, Temecula, CA), GFAP (an astrocyte marker; 1:1000; Chemicon), and OX42 (CD11b, a microglial marker; 1:200; Santa Cruz Biotechnology, Heidelberg, Germany) over 2 nights at 4°C, followed by a mixture of Cy2- and Cy3-conjugated secondary antibodies for 1 hour at room temperature. The double-stained images were examined with an Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan). The method to quantify spinal microglial activation is provided in the following text.

Quantification of Spinal Microglial Activation

Assessment of microglial response was performed by measuring OX42 immunoreactivity in 7 to 12 sections chosen at random from the required segments of the spinal cord. The area of OX42 immunoreactivity under 100× magnification was measured in the superficial dorsal horn of the spinal cord. Analog images of the spinal cord were captured via video link to an Olympus BX61 microscope and converted into a digital monochrome image by a computer-assisted image analysis system (Image J; NIH Image, National Institutes of Health, Bethesda, MD) with gray scales ranging from 0 to 255. The gray-shade detection threshold was set at a constant level to allow detection of positive immunostaining, and the area of highlighted immunoreactivity was expressed as a percent of the area of the field scanned.1,2

Supplementary References

Supplementary Figure 1. Microglial activation (OX42 expression) in the thoracic, lumbar, and sacral segment of the spinal cord at 21 days after TNBS or vehicle instillation into the pancreatic duct. The intensity of OX42 immunoreactivity in the superficial dorsal horn was significantly higher when receiving direct afferent input from the pancreas (T8–T12) but was also observed in the neighboring segments. TNBS vs vehicle: T1 and S2, \( P > .05 \); T2–T4 and S1, \( P < .05 \); the remaining segments: \( P < .01 \).

Supplementary Figure 2. Spinal microglia activation (OX42 expression) at different time points after TNBS or vehicle instillation into the pancreatic duct. The intensity of OX42 immunoreactivity in the T12 superficial dorsal horn was significantly higher at all 3 time points (D1, D7, and D21) in the TNBS-treated than in the vehicle-treated group. The intensity of OX42 immunoreactivity gradually increased at D7 and reached the maximum at D21 after CP. \(* P < .001\), vehicle group vs TNBS group (independent-sample t test); \#P < .001, D21 vs D1 in TNBS group. D, day.

Supplementary Figure 3. Spinal P-p38 levels showed a significant positive correlation with nociceptive behaviors in response to the von Frey stimulus (\( r = 0.673; P < .001 \)).