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Peripheral Neuritis Trauma in Pigs: A Neuropathic Pain Model

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Abstract: **The use of rodents in preclinical studies has contributed greatly to our understanding of the pathophysiology of chronic neuropathic pain. These animal models are limited because of their poor clinical translation. We developed a pig model for chronic pain caused by surgically induced peripheral neuritis trauma (PNT). Seventy-five percent of the animals exhibited mechanical and tactile allodynia, which are indicative of painful neuropathy, by day 28 after surgery. Importantly, the PNT-injured pigs retained their ability to walk or to stand on their injured leg. Messenger RNA analysis of acute inflammatory cytokines calcitonin gene-related peptide and brain-derived neurotrophic factor at the site of injury suggests transient inflammation followed by a persistent high level of neurologic markers. Gabapentin and morphine effectively inhibited hypersensitivity to von Frey filaments and to feather stimuli, and reversed spontaneous pain-related behavior in a dose-related manner. No analgesic effect was detected in PNT-injured pigs after treatment with aprepitant, similar to observations in humans and contrary to observations in rodents. In conclusion, PNT-induced trauma in pigs may comprise a valid preclinical model for the study of the chronification of peripheral nerve injury and for the study of new pain therapies.**

Perspective: ***This article presents the characterization of a new peripheral neuritis trauma (PNT) model in pigs. The pig PNT model could help close the translational gap between preclinical and clinical responses and may contribute to improved efficacy or safety of candidate drugs.***

Key words: *Sciatic nerve trauma, pain chronification, pain behavior, CX3CR1, brain-derived neurotrophic factor.*

Nerve injury in the peripheral or central nervous system can result from various insults, including trauma (either from an accident or from surgery), as well as from inflammatory-medicated or immune-mediated processes. Rodent models of traumatic nerve injury are commonly used for research because of their reproducibility and simplicity.[49] The rat sciatic nerve crush injury model is widely used to assess posttraumatic impairment of motor function.[3,33,47] Peripheral nerve injury methods are also commonly used in the rat. These include, for example, loose ligation of the whole peripheral nerve, known as chronic constriction injury[4]; ligation of a section of a large peripheral nerve or partial sciatic nerve ligation[39]; ligation of the L5 and L6 spinal nerves, also known as spinal nerve ligation[19]; and spared nerve injury, in which 2 of the 3 terminal sciatic branches are cut.[9,35] Rodent models have led to a substantial increase in knowledge of pain mechanisms over the last few decades. However, 1 of their major shortcomings is the frequent failure to predict drug efficacy in humans.[30] The most prominent example of efficacy-related translational failure is with substance P neurokinin 1 (NK-1) antagonist, aprepitant (MK-869).[13] Preclinical evaluation of potential analgesic drugs in higher animal species might contribute to an improvement in translational efficacy.

We developed a peripheral neuritis trauma (PNT) model in an attempt to address the gap between rodent studies and human studies. The pig was chosen for this work because it is considered an excellent model for human disease and exhibits anatomic, physiologic, and neurologic resemblance to humans.[44] Pig skin is known

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for its close physiologic resemblance to human skin, including the function and structure of the nerves as well as the skin innervation.[10,21,44] The skin is the end organ for testing hyperalgesia and allodynia responses, and pigs respond to such tests in a manner that reflects human responsiveness.[5] Given the greater anatomic and neurologic resemblance of pigs to humans, compared with rodents, we hypothesized that the pig PNT model might comprise a useful contribution for understanding pain and for testing new pain therapies. A pig model will also increase the feasibility for evaluating new devices that cannot be evaluated on rodents because of their small size.

The main objective of this work was to characterize the sciatic nerve trauma-induced neuropathic pain model. The benefits and limitations of the model were evaluated based on 3 main categories: 1) behavioral changes, including reflexive hyperalgesia and allodynia measures, operant spontaneous expression, and motor function changes consistent with disturbed pain integration; 2) biomarker and histology analyses of the site of injury and spinal cord; and 3) evaluation of the pharmacologic relevance of this model by assessing the effectiveness of morphine, gabapentin, and aprepitant. The results were anticipated to establish the pig PNT model as a valuable tool for translational efforts that will facilitate the development of neuropathic pain treatments.

Methods

Animals and Housing

Danish Landrace X Large White crossbred pigs from the domestic herd at Lahav Laboratories, Negev, Israel, were used in this study. All procedures and experiments were approved by the institutional animal care and use committee and were designed to minimize the number of animals as well as undue suffering in accordance with the International Association for the Study of Pain.[56] Before the study, all animals were kept under conventional pig production conditions. The animals were housed in open pens (1.4 × 2.4 m) on a 12 h/12 h light/dark cycle 7 days before the start of the study. Feeding occurred 3 times daily using special pig food (Dry Sows; Milobar, Oshrat, Israel). The pigs were provided with opportunities to root and chew for enrichment. Fresh water was provided ad libitum by an automated system.

Habituation

The pigs were habituated to the study protocol for 5 days before surgery, as described previously.[5] The pigs were trained to walk to the preparation room daily during the habituation period to familiarize them with the schedule and the technicians. They were always returned to their original pens with their original penmates. The habituation process was conducted to reduce the pigs' stress level. The temperature in the surgery room was maintained at 19°C (range = 18°C–20°C). The animals were weighed at 6 time points: at the beginning of the acclimatization period, 5 days before surgery (study day –5), on the day of surgery (study day 0) before anesthesia, and after surgery on study days 7, 10, 18, and 28.

Anesthesia and Surgery

Each pig walked freely to the preparation room on the day of surgery. A technician carried the animal in his hands and placed an anesthetic facemask (Akzent Color; Fritz Stephan, Gackenbach, Germany) on the pig's mouth and nose, as described previously.[5] Each animal was anesthetized with a 3% isoflurane/100% oxygen mixture. The technician held the pig until it was relaxed and sleepy. It was shaved and swabbed with 70% ethanol, and was immediately carried to the operating room. The pig was placed in the sternal position on the operating table. The incision area was swabbed with antiseptic liquid polydine solution (Polysept Solution; Rekah Pharmaceutical Industry Ltd., Holon, Israel) and the nonoperated areas were covered with sterile sheets. Blood O_2 saturation was monitored for the duration of anesthesia (Spacelab Medical, Snoqualmie, WA, USA).

Figure 1 (A and B) shows the location of the sciatic nerve and the trauma methodology. An incision of 8 to 10 cm was made through the skin and fascia on the left side of the lower back, toward the caudal end, and approximately 1.5 cm lateral and parallel to the spine line of the pig. The muscles were then retracted and the entire sciatic nerve was exposed. PNT was induced by 3 silk threads (3-0; Assut, Huddersfield, UK), each

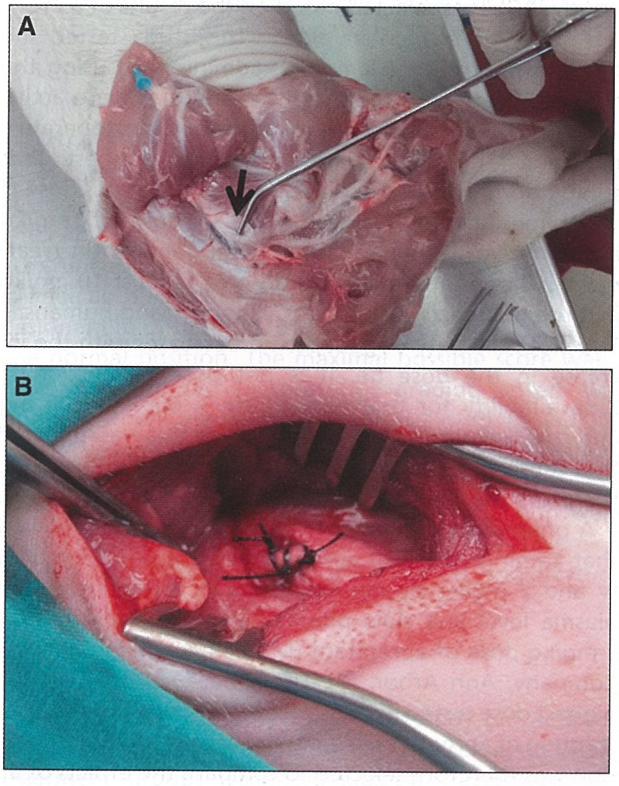

Figure 1. Sciatic nerve trauma methodology. **(A)** Postmortem illustration of injury location. The black arrow shows the area of sciatic nerve injury that innervates the knee and the foot. Note the avoidance of the hind leg innervation. **(B)** Lateral half of the sciatic nerve bundle immediately after PNT, showing 3 loose ligations (1–2 mm apart).

3 cm in length, that were presoaked overnight in complete Freund's adjuvant (CFA) (1 mg/mL). After sciatic nerve exposure, the presoaked threads were used to create 3 loose ligations (1–2 mm apart) surrounding the lateral half of the sciatic nerve bundle (Fig 1B).

PNT was induced using threads presoaked in CFA, an inflammatory agent widely used in animal chronic pain models.[25] Performing the same procedure without CFA did not result in persistent pain.

A control group (n = 6; sham group) underwent anesthesia and skin incision only, but the sciatic nerve was left intact.

Wound Closure and Postsurgery Treatment

Incisions were closed using a 2-layer method. The subcutis layer was first sutured with vicril 3-0 continuous stitches. The skin was then closed with a continuous suture using a 3-0 silk thread (Assut). After the incision, all pigs received marbofloxacin (10% w/v) (Marbocyl; Vétoquinol UK Ltd., Buckingham, UK) administered via intramuscular injection into the neck muscle at a total dose of .5 mL per pig. This treatment was continued for 5 consecutive days. After recovering from anesthesia, the animals were returned to their home pen for recovery.

Drugs

Morphine (Teva Pharmaceutical Industries Ltd., Petach Tikva, Israel), at doses of .1 mg/kg and .3 mg/kg (intravenously [IV]), was selected based on the results of a previous study performed in pigs.[27] This study tested the effect of morphine (.1 mg/kg), administered using the epidural route, and reported that the pigs were active with no differences in preoperative and postoperative behavior. The data from this study suggest that stimulation-produced analgesia is avoided at this dose.[27]

Gabapentin (USP, Rockville, MD, USA) doses of 3 mg/kg and 6 mg/kg (IV) were chosen. Oral gabapentin treatment is frequently used at a dose of at least 10 mg/kg in large mammals such as cats,[40] dogs,[20] and humans.[52] The bioavailability of gabapentin decreases with the increase in the dose (ie, it is not dose proportional). Bioavailability for oral gabapentin is 60% at 300 mg and ≤40% at doses of 1600 to 4800 mg.[28] It was reported that cats responded to 4 mg/kg IV gabapentin,[40] and we therefore used an initial gabapentin dose of 3 mg/kg IV.

A pharmacokinetic study carried out in dogs showed that administration of aprepitant at a dose of 1 mg/kg IV and 2 mg/kg by mouth results in a relatively high plasma level and slow plasma clearance.[15] We used a 2-mg/kg dose of aprepitant (MK-869; Cayman Chemical Company, Ann Arbor, MI, USA), because this was the highest dose tested in dogs. Little is known about the absorption of aprepitant in pigs. The IV route of administration was therefore selected to compare the effects of all 3 drugs tested in the PNT model.

Study Design

A total of 30 male pigs were used in this study, after selection. The animals that were selected on study day 7 exhibited changes in behavior and reduction in withdrawal threshold after von Frey (vF) testing. Seventy-five percent of the animals experienced these changes. These 2 parameters were chosen as inclusion criteria because we have knowledge of pigs' behavior after a painful procedure and know the range of their responses to vF. To the best of our knowledge, the feather test had not previously been tried in pigs. The inclusion criteria could thus not rely on a new unknown readout.

All pigs were 9 to 10 weeks old, weaned, and weighed 13 ± 1 kg at the start of the study. The study included 5 phases: 1) habituation (study days −5 to −1); 2) surgery (study day 0); 3) follow-up (study days 1–28); 4) termination (study days 10 and 28); and 5) effect of drug treatment: morphine, gabapentin, or aprepitant (study days 28 and 30). Table 1 summarizes the study design and the animals' assignment to the treatment groups. No pain assessment was performed on study day 3 because of animal hypersensitivity after incision, as reported previously.[5] Expression of spontaneous pain and motor function were scored on study days −1 and 7, 10, 18, and 28 after surgery. Three animals were culled at 2 time points (study days 10 and 28), and the spinal cord (L5–S1) and sciatic nerve tissue (1 cm area of injury) were collected for RNA analysis.

Assessment of Drug Efficacy in the PNT Model

On study day 28, after verification that the animals still responded to pain, the pigs were divided into 4 groups (Table 1): group 1 received saline on day 28 and 48 hours later; group 2 received gabapentin initially and after 48 hours (3 mg/kg and 6 mg/kg IV, respectively); group 3 received aprepitant (2 mg/kg, IV); and group 4 received morphine initially and after 48 hours (.1 mg/kg and .3 mg/kg IV, respectively).

All assessments of hyperalgesia and allodynia, spontaneous pain expression, and motor function were monitored at the following time points: before dosing and 1, 3, and 5 hours after dosing. The animals were weighed using a standard balance: immediately after the behavior assessments on study day −5 (just before the beginning of the acclimatization and habituation period), on study day 0 (before surgery), and on study days 7, 10, 18, and 28 (Table 1).

Pain Assessment Methods

Mechanical Sensitivity Test Using the vF Method

Mechanical sensitivity was assessed using vF filaments (von Frey Touch Test, Sensory Evaluator Kit, model 58011; Stoelting Co, Wood Dale, IL). The tests were performed in the pigs' home pen. vF filaments ranging from a minimum of 1 g (diameter = .229 mm, force = 9.804 mN) to a maximum of 60 g (diameter = .711 mm, force = 588.253 mN) were used. Each filament was applied for 1 to 2 seconds on the dorsal area of the animals' foot and on the external side of the knee. The filaments were applied 3 times with a 5-second to 10-second interval between applications,

Table 1. Summary of Study Design and Animal Disposition

Study Day	Assessment	Number of Animals, n			
−5	BW	30			
−1	BW/vF/F/SB/MF	30			
0	BW/S	30			
7	BW/vF/F/SB/MF	30			
10	BW/vF/F/SB/MF	30			
	TH	3			
18	BW/vF/F/SB/MF	27			
28	TH	3			
	BW/vF/F/SB/MF predose/R	24			
	Drug Treatment Groups (N = 24)				
	Study drug and dose	Saline	Morphine .1 mg/kg IV	Aprepitant 2 mg/kg IV	Gabapentin 3 mg/kg IV
	n	6	6	6	6
	vF/F/SB/MF at 1, 3, and 5 h postdose	6	6	6	6
30	BW/vF/F/SB/MF predose	6	6	6	6
	Study drug and dose	Saline	Morphine .3 mg/kg IV	N/A	Gabapentin 6 mg/kg IV
	n	6	6		6
	vF/F/SB/MF at 1, 3, and 5 h postdose	6	6	N/A	6

Abbreviations: BW, body weight; vF, von Frey test; F, feather test; SB, assessment of spontaneous behavior; MF, motor function assessment; S, surgery; TH, tissue harvesting; R, randomization of animals to groups; N/A, not applicable.

using the up-down method.[8] If withdrawal was not achieved, a thicker filament was applied. If withdrawal was achieved, a thinner filament was applied. Alternating the filaments enabled determination of the threshold required to achieve a withdrawal reaction. This procedure was carried out at the following time points: 1 day before surgery (study day −1), and on study days 7, 10, 18, and 28 (Table 1).

Tactile Sensitivity Test Using a Feather Stimulus

The tactile stimulus consisted of a 12.5-cm pigeon feather, which delivered light tactile stimulation. The feather was gently stroked on the dorsal area of the animals' foot. Responders included animals expressing all 3 of the following behaviors: moving away, shaking and keeping the leg up, and guarding the leg for a period of 5 seconds. The feather test was conducted 1 day before surgery (study day −1), and on study days 7, 10, 18, and 28 (Table 1).

Assessment of Spontaneous Pain Behavior Using a Composite Behavior Scale

The solitary performance and social behavior of each animal were scored during a 10-minute observation period. Seven behavioral parameters were observed and recorded (3 for solitary performance and 4 for social behavior). The animals were observed in their home pen by the caretaker who handled them from the first acclimation day. Table 2 summarizes the behavioral parameters and the corresponding scoring method. In general, the parameters relate to observing the animals' standing posture, leg guarding, leg shaking, as well as their vocalization and social behavior (isolation and aggressiveness).[37] Some parameters were not so common and therefore carry more weight because they indicate more pain (eg, vocalization changes are less common than changes in weight bearing).

Each parameter was graded from 0 to 2, depending on the observed behavior. The sum of all points from the 7 parameters was considered the final score. A higher score indicated that the animal expressed more spontaneous pain behavior. The maximum possible score was 11 points. Spontaneous expression scores were recorded 1 day before surgery (study day −1), and on study days 3, 7, 10, 18, and 28.

Motor Function Assessment

The ability of the pigs to use their leg properly was assessed by observing the animals' standing posture and their ability to walk properly. Table 3 summarizes the motor function parameters tested. Motor function was graded as follows (from 0 to 2 points): 0 = normal; 1 = occasional flip of the foot; 2 = not able to keep the foot in the normal position. The maximal possible score was 4 (severe motor dysfunction). This grading system was applied when the animals were standing as well as when they were walking. Animals experiencing secondary wounds (eg, to the toenails or to the dorsal area of the foot) as a result of a loss of motor function were culled for ethical reasons. Motor function scores were recorded 1 day before surgery (study day −1), and on study days 3, 7, 10, 18, and 28.

Blinding Method

The study was performed as a blind study. Before treatment, the animals were randomized and assigned to 1 of 4 treatment groups (saline, gabapentin, aprepitant, or morphine). The technicians who prepared the drugs were blinded as to the treatment group assignments for the duration of the study. The veterinarian who administered the drugs received coded vials and remained unaware of the treatment given to individual animals.

Table 2. Behavior Parameters and Scoring Method

Category	Parameter Tested	Description of Behavior	Score
Solitary performance (maximum score = 4)	Weight bearing	Equal on both legs	0
		Carrying weight mainly on intact leg	1
	Appearance	Normal lying and walking	0
		Guarding the injured leg	1
	Vocalization	Normal vocalization (low volume)	0
		High-volume occasional cry	1
		Screaming and cries	2
Social behavior (maximum score = 7)	Restlessness	Normal behavior	0
		Pacing around the pen	1
		Jumping up and down and pacing around the pen	2
	Agitation	Normal behavior	0
		Slightly moves away when approached	1
		Screaming and moves away when approached	2
	Aggression	Friendly	0
		Moves away	1
		Attacking and biting his penmates	2
	Isolation	Normal behavior	0
		Moves away from penmates	1

NOTE. The scoring criteria used are based on a numerical rating scale modified from Reyes et al (2002).[37] The behavior score was divided into 2 distinct categories: 1) solitary performance and 2) social behavior. The total score is the sum of all subscores.

Biomarker Analysis Using Quantitative Polymerase Chain Reaction

Three animals were humanely euthanized according to animal welfare guidelines on study days 10 and 28, and the sciatic nerves (.5 cm area of the site of injury) and spinal cords (L5–S1) were dissected on ice. The ipsilateral spinal cord, contralateral spinal cord, and the sciatic nerve were extracted and evaluated for messenger RNA (mRNA) expression of brain-derived neurotrophic factor (BDNF) and fractalkine receptor (CX3CR1). BDNF and CX3CR1 are markers for neuropathic pain and microglia activity.[17,45] Total RNA was extracted using a Navy Bead Lysis Kit (Next Advance Averill Park, NY) and the Maxwell 16 LEV Simply RNA Tissue Kit (Promega, Madison, WI) according to the manufacturer's instructions. After the RNA was extracted, it was quantified using a Qubit RNA Broad Range Kit (Life Technologies, Grand Island, NY). Quality control was performed using TapeStation 2200 RNA ScreenTape, ladder, and reagents (Agilent Technologies, Santa Clara, CA), according to the manufacturer's instructions. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY), using random primers, according to the manufacturer's instructions. Preamplification of cDNA was performed using the TaqMan PreAmp Master Mix (Life Technologies, Grand Island, NY), according to the manufacturer's instructions. A quantitative polymerase chain reaction (probe) for gene expression was performed using predesigned assays for calcitonin gene-related peptide (CGRP) (Ss03386432_uH), BDNF (Ss03822335_s1), tumor necrosis factor α (TNF-α) (Ss03391316_g1), interleukin 1β (IL-1β) (Ss03393804_m1), as well as custom-designed assays for CX3CR1 and β-actin (Life Technologies, Grand Island, NY). TaqMan Fast Advanced Master Mix (Life Technologies, Grand Island, NY) was used according to the manufacturer's instructions. The general bioinformatics analysis was performed using Life Technologies Data Assist Software (Grand Island, NY).

Table 3. Motor Function Parameters and Scoring Method

Category	Parameter Tested	Description of Behavior	Score
Motor function (maximum score = 4)	Walking	Normal	0
		Flipping the foot occasionally	1
		Flipping the foot all the time	2
	Standing	Normal	0
		Flipping the foot occasionally	1
		Flipping the foot all the time	2

NOTE. The scoring criteria used are based on a numerical rating scale modified from Reyes et al (2002).[37] The behavior score measured 2 distinct parameters: 1) walking and 2) standing. The total score is the sum of all subscores.

Histology Analysis

Three animals were humanely euthanized according to animal welfare guidelines on study days 10 and 28 and the sciatic nerve (.5 cm area of the site of injury, including at least 1 suture) was removed for histology observations. The individual samples were immediately pinned to a flat piece of polystyrene to maintain their shape and were placed in plastic histology cassettes with 10% neutral buffer formalin (4% formaldehyde) for 72 hours at room temperature. The tissue samples were then processed routinely for light microscopy by dehydrating, embedding, and cutting.[2] The samples were cut into 5-μm transverse increments with a microtome and stained with hematoxylin and eosin according to standard procedures.[2]

Table 4. Pain Assessment Before Drug Treatment

	Study Days Before Dosing				
Method of Assessment	−1 (Before Surgery)	7	10	18	28
PNT animals					
n	30	30	27	27	24
vF test, withdrawal force, *g*	60.00 ± 0	1.40 ± .49*,**	2.00 ± 1.20*,**	1.57 ± 1.20*,**	2.07 ± 1.51*,**
Feather test, % responders	0	83.33*,**	100.00*,**	100.00*,**	83.33*,**
CBS, mean group points	0 ± 0	6.17 ± 2.14*,**	7.17 ± 2.64*,**	6.67 ± 2.66*,**	5.75 ± 1.41*,**
MF, mean group points	0 ± 0	.33 ± .82	0 ± 0	0 ± 0	.17 ± .41
Sham animals					
n	6	6	6	6	6
vF Test withdrawal force, *g*	60.00 ± .00	54.33 ± 5.67	60.00 ± 0	60.00 ± 0	60.00 ± 0
Feather test, % responders	0	0	0	0	0
CBS, mean group points	0 ± 0	.3 ± .2	0 ± 0	0 ± 0	0 ± 0
MF, mean group points	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Abbreviation: MF, motor function scale.
*$P < .05$ vs values measured on day (−1) before surgery.
**$P < .05$ vs sham animals.

Statistical Analysis

Behavioral score data were analyzed using appropriate models of analysis of variance (ANOVA) (ie, repeated measures ANOVA), and Scheffé correction for multiple comparison *P* values. This model was not suitable for motor function and vF analysis. Nonparametric tests were applied for motor function analysis (exact Kruskal-Wallis test). No corrections were made for multiple compressions. Fisher's exact test was performed for the feather test. All data are presented as the mean ± standard deviation (SD). mRNA data are presented as mean fold over control (naive animals) ± SD. A *P* value of <.05 was considered significant.

Results

All animals gained weight during the study. At the start of the habituation period (study day −5), the mean weight of the animals was 11.22 ± .69 kg. Five days later, on the day of surgery (day 0), the mean weight of the animals remained unchanged (11.32 ± .76 kg). This is typical for this period. The weight of the animals increased during the study, and on study day 10, their mean weight was 13.05 ± .77 kg. At the end of the study, the animals gained approximately 37% body weight. All animals were active and walked normally.

Pain Assessment

On study day 7, 75% of the pigs showed symptoms of pain, which were expressed in a reduction in the withdrawal threshold after vF testing and an increase in the behavior score (Composite Behavior Scale [CBS]). Only these pigs were included in the study.

Pain Assessment Before Drug treatment

After surgery, the animals showed high sensitivity to vF stimuli, high sensitivity to light touch (feather test), and significant changes in spontaneous behavior (Table 4). On study day 28, 1 hour before saline treatment, the mean group withdrawal threshold after vF filament application was significantly lower than the values recorded before surgery (mean group withdrawal: 2.07 ± 1.51 g vs 60.00 ± 0 g, respectively; $P < .01$) (Table 4). Five of the 6 animals assigned to the saline group expressed withdrawal behavior after the feather test (83.33%). The mean group behavior score just before drug treatment was 5.75 ± 1.41 points. During the 10-minute observation period, the mean group motor function score remained low (.17 ± .41 points; Table 4). Sham-operated animals did not express a decrease in withdrawal threshold or significant changes in the CBS.

Animals that underwent ligation with threads without CFA demonstrated a reversible decrease in the withdrawal force after vF testing. The baseline mean group withdrawal force of these animals was 60.00 ± 0 g. Seven days after the manipulation, the mean group withdrawal force was 2.50 ± .19 g. This value was significantly lower than the baseline ($P < .05$). However, 10 days after surgery, the withdrawal threshold was 31.51 ± 8.22 g and this was not significantly different from the mean group baseline withdrawal threshold value. Testing the withdrawal force again on study day 18 revealed a further mean group increase in the withdrawal threshold (47.55 ± 6.52 g). Only 1 of these 6 animals responded to the feather test and their changes in behavior, although significant, were mainly expressed in their ability to carry weight on the operated leg.

The Effect of Drugs on Response to Mechanical Stimulation (vF Test)

Figure 2 shows the effect of saline, gabapentin, aprepitant, and morphine on PNT pigs' response to vF testing. Treatment with gabapentin (IV) at doses of 3 mg/kg and 6 mg/kg resulted in a dose-related response 1 hour after injection (mean withdrawal: 15.00 ± 8.56 and 35.5 ± 19.45, respectively; $P < .05$ vs pretreatment values).

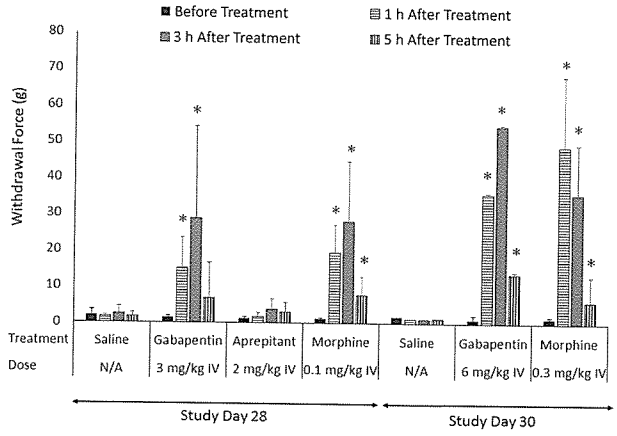

Figure 2. Effect of drugs on PNT pigs' response to vF testing. The force (*g*) required to achieve a withdrawal response on the dorsal side of the foot was measured before drug treatment and at 1, 3, and 5 hours after dosing on study days 28 and 30. Values are presented as the mean ± SD. *$P < .05$ versus predosing on each day of testing. The number of animals tested in each group on both days studied was 6. *The mean group baseline withdrawal force before surgery was 60.00 ± 0 *g*.

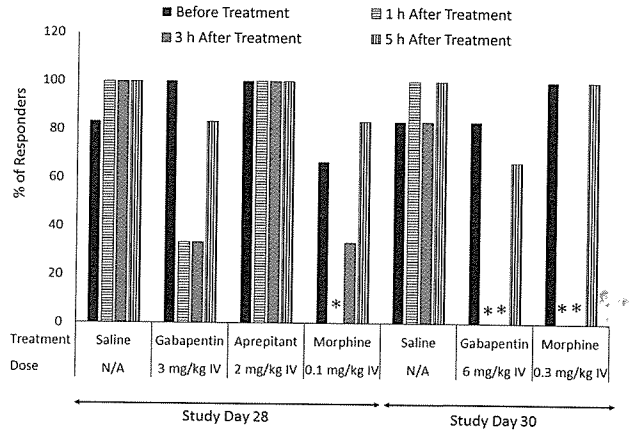

Figure 3. Effect of drugs on PNT pigs' response to feather stimulation. Responders included animals expressing all 3 of the following behaviors: moving away, shaking and keeping the leg up, and guarding the leg for a period of 5 seconds. Response was measured before drug treatment and at 1, 3 and 5 hours after dosing on study days 28 and 30. Values are presented as the % of responders. The number of animals tested in each group on both days studied was 6.

Treatment with aprepitant (IV) at 2 mg/kg did not affect the withdrawal threshold response. Subjective observations found that 2 of the 6 animals dosed with aprepitant expressed marked aggressiveness, such as trying to bite their technician.

At 1 hour after morphine dose, the withdrawal threshold increased in a dose-related manner. The mean group withdrawal threshold 1 hour after morphine dose of .1 mg/kg (IV) was 19.33 ± 7.74 g. The mean group withdrawal threshold 1 hour after morphine dose of .3 mg/kg (IV) was 48.67 ± 17.56 g ($P < .05$ vs before treatment). The withdrawal threshold was reduced 5 hours after dosing (Fig 2).

The Effect of Drugs on Response to Tactile Stimulation (Feather Test)

Figure 3 shows the effect of saline, gabapentin, aprepitant, and morphine on PNT-injured pigs' response to light touch (feather testing). At 1 hour after gabapentin dosing (3 mg/kg IV), 2 of the 6 animals responded to tactile stimulation (33.33%). Five hours after treatment, the number of animals that responded was the same as before treatment (ie, 5 of 6 animals; 83.33%). Forty-eight hours later (study day 30), the animals were given 6 mg/kg gabapentin (IV). None of these animals responded to the feather stimuli after 1 or 3 hours (Fig 3). Five hours after dosing (6 mg/kg gabapentin), 4 of the 6 animals responded to the feather stimuli (66.67%).

Animals treated with aprepitant (2 mg/kg IV) responded to the feather stimuli at each time point measured.

After treatment with morphine (.1 mg/kg IV), none of the animals responded to feather stimuli at 1 hour after dosing. Two of 6 animals responded to the test 3 hours (33.33%) and 5 hours (83.33%) after dosing. Treatment with the higher morphine dose (.3 mg/kg IV) resulted in complete attenuation of the response to feather stimuli for 3 hours (Fig 3).

Effect of Drugs on Spontaneous Pain Behavior and Motor Function Scores

After treatment with gabapentin (3 mg/kg), a reduction in spontaneous expression of pain was observed at 1 and 3 hours after dosing (Fig 4). The higher dose of gabapentin (6 mg/kg) resulted in a further reduction in spontaneous behavior. At the higher dose, 4 of the 6 animals behaved normally but 2 animals exhibited modest behavioral changes. The group mean score for 6 mg/kg gabapentin was .50 ± .84 points at 3 hours after treatment, which differed significantly from the group mean score before treatment (5.50 ± .84 points, $P < .05$) and from the saline-treated group mean score at 3 hours after treatment (5.67 ± 1.97 points, $P < .05$).

Animals treated with aprepitant expressed significant changes in behavior and scoring the changes was challenging. Aprepitant-treated animals showed aggressive behavior: 2 pigs tried to bite their penmates as well as the technician during the observation period.

Morphine treatment resulted in a significant decline in spontaneous pain behavior. The higher dose tested in this study (.3 mg/kg IV) resulted in an almost complete inhibition of spontaneous behavior at 1 and 3 hours after dosing (.50 ± .55 at both time points, $P < .05$ vs predosing as well as vs saline). Five hours after dosing, the effect of morphine was no longer observed.

All animals tested were active, walking, and approached the food and water. Separate motor function analysis revealed that motor dysfunction and paw flip were minimal (see Fig 5).

Histology of the Site of Injury

Sciatic tissue at the site of injury was collected on study days 10 and 28 from culled pigs with induced PNT injuries. The histology observations suggest that similar changes were visible in all PNT samples but to a variable degree. The changes observed were granulomatous

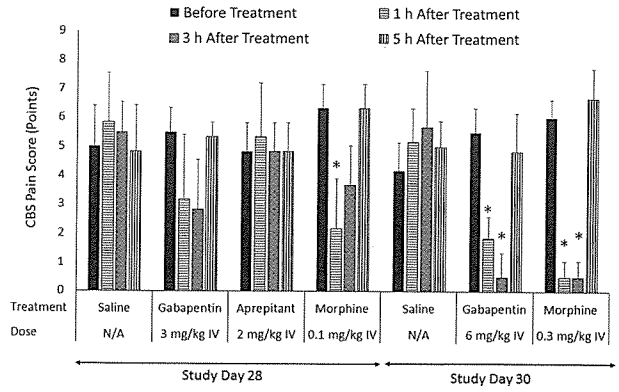

Figure 4. Effect of drugs on spontaneous pain-related behavior using a CBS in PNT pigs. CBS monitored before treatment and at 1, 3 and 5 hours after dosing on study days 28 and 30. Values are presented as the mean ± SD. *$P < .05$ versus predosing on each day of testing. The number of animals tested in each group on both days studied was 6.

inflammation and degeneration of peripheral nerves with features typical of Wallerian degeneration.[14] Mixed acute and chronic inflammation was also visible, which is typical of persistent inflammation. As a rule, inflammation occurs in connective tissue surrounding the nerves, as can be seen, for example, in Fig 6, sample 70B, panels K and L. Endoneurial inflammation was rare. The physical proximity of the nerve bundles to the site of inflammation is the main, but not the sole, factor responsible for their degeneration, as can be seen, for example, in Fig 6, sample 69A, panels F and G. On study day 28, Schwann cell hyperplasia (SCHP) was frequently observed, for example in Fig 7, sample 76A, panels E1 and E2.

mRNA Analysis

Gene Expression Analysis of Spinal Cord

Spinal cord tissue extracted on study days 10 and 28 was analyzed for mRNA expression of BDNF and CX3CR1 (Table 5). The mRNA levels of animals that underwent a nerve trauma procedure were calculated as mean fold over naive animals. On study day 10, the ipsilateral CX3CR1 expression level was more than 2-fold higher in PNT animals compared with the contralateral side (2.21 ± .28 vs 1.13 ± .06, respectively; $P < .05$).

On study day 28, there were no side-specific differences, and both sides (ipsilateral as well as contralateral) expressed relatively low levels of CX3CR1. On this day, BDNF expression in the ipsilateral spinal cord in the PNT animals was significantly increased by almost 5.5-fold (5.53 ± 1.13) over the naive animals. This increase was significant compared with the contralateral side (.80 ± .30, $P < .05$). No significant changes in BDNF were found in the early stages of the injury (study day 10; Table 5). No significant changes were recorded in sham-operated animals.

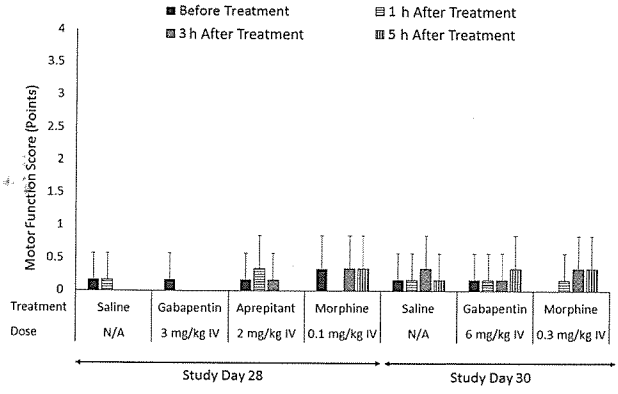

Figure 5. Effect of drugs on the motor function (MF) score in PNT pigs. MF monitored before treatment and at 1, 3 and 5 hours after dosing on study days 28 and 30. Values are presented as the mean ± SD. *$P < .05$ versus predosing on each day of testing. The number of animals tested in each group on both days studied was 6.

Gene Expression Analysis of Injury Site

Analysis of gene expression in the sciatic nerve at the site of injury is shown in Table 6. A significant increase in TNF-α and IL-1β levels was measured on study day 10 for PNT (5.03 ± 1.19 and 5.94 ± 1.59 fold, respectively). By study day 28, the expression levels of TNF-α and IL-1β had decreased significantly compared with expression levels on study day 10 ($P < .05$) (Table 6).

BDNF expression levels at the site of injury in PNT animals were significantly increased by almost 200-fold (193.47 ± 67.01) over control levels on study day 10. On study day 28, BDNF expression was reduced compared with study day 10, but the levels detected remained high, exceeding 100-fold (127.75 ± 31.22), versus control animals.

Expression of CGRP mRNA at the sciatic injury site was also increased on study day 10 (10.36 ± 4.00-fold). On study day 28, CGRP mRNA expression was reduced (Table 6).

Discussion

The aim of this work was to develop a translational model for trauma-induced neuropathic pain. After PNT-induced trauma, the pigs developed sustained high sensitivity to mechanical stimulation and clear mechanical allodynia, exhibited detectable spontaneous behavior changes, and had minor to nonexistent motor dysfunction. Histology of the site of injury suggests a mix of damaged bundles and intact bundles. mRNA analysis shows the presence of an inflammation phase followed by a neurogenic phase, and spinal cord mRNA analysis shows an early and transient increase in a microglia marker followed by a late increase in BDNF. All behavior parameters were altered after treatment with gabapentin or morphine. Aprepitant failed to relieve any of the pain-related symptoms.

Most studies on pain mechanisms and pharmacology are conducted on rodents. Five main criticisms of rodent models are that pain models in animals assume sciatic nerve neuroanatomic similarities between species and strains[38]; excessive emphasis is placed on reflexive withdrawal from a stimulus, neglecting the fact that the primary symptom of chronic pain in humans is spontaneous pain[31,32]; existing models are too artificial, with

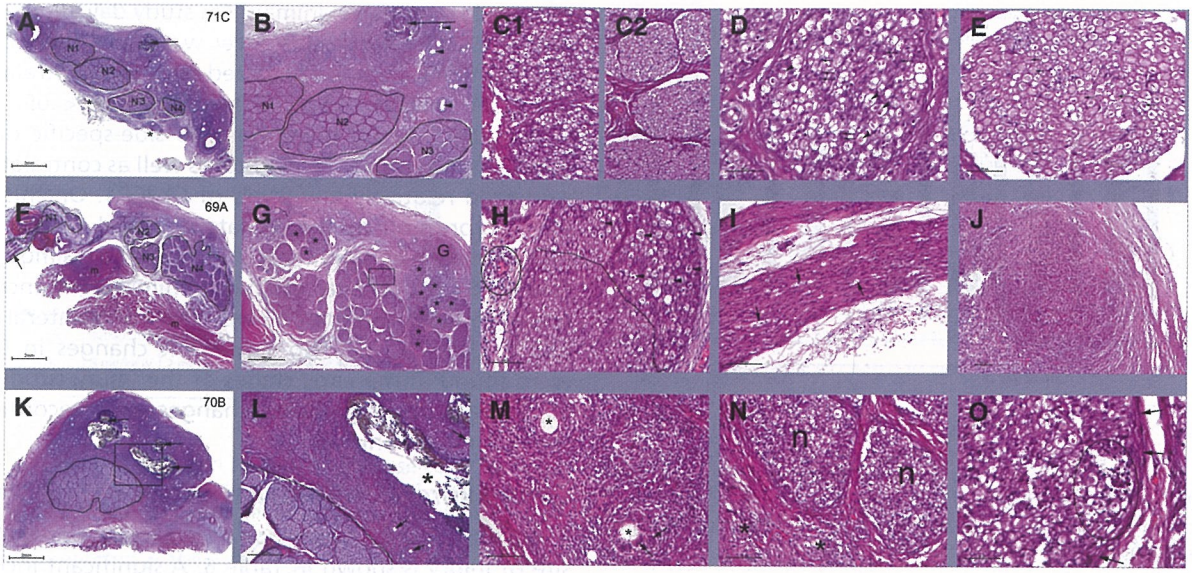

Figure 6. Histology samples from PNT pigs (study day 10). Sample 71 C: **(A)** Low magnification. Severe inflammation is visible as dense, cellular, bluish tissue, surrounding 4 nerve bundles (marked N1–N4). Inflammation is most pronounced near the suture (arrow) and least severe furthest from the suture, along the lower margin (asterisks). **(B)** Medium magnification of the central region. This field includes 3 nerve bundles (marked N1–N3). There is widespread degeneration in N1 and N2. Nerve bundle N3 is essentially within normal limits (WNL). At this magnification, the difference is barely noticeable. However, N1 and N2 are more granular/cellular than N3. (C) Side-by-side comparison of degenerate (C1) and normal (C2) nerve fascicles. **(D)** High magnification of a transverse section of a nerve fascicle showing degenerative changes of N1 and N2 in this sample. There is widespread swelling of axonal sheaths, many of which contain eosinophilic debris or macrophages (arrowheads). Other axonal sheaths are empty. Multifocal mild SCHP is present (arrows). A few residual axons may be present, but none was clearly identified. Compare this appearance with **(E)**. **(E)** High magnification of a transverse section of a normal nerve fascicle from N3. The nerve fascicle is composed of a collection of axons with a minimal amount of intervening tissue (endoneurium). A few well-preserved axons are indicated (arrows). Sample 69A: **(F)** low magnification. Inflammation is mild and mostly limited to the upper field of the sample. Four nerve bundles are indicated (N1–N4). The area indicated by an arrow is shown at high magnification in I. **(G)** Medium magnification of the central region. In general, nerve fascicles close to the site of inflammation show degenerative changes (asterisk), whereas those located further away are WNL. G = granuloma (shown at high magnification in **(J)**). The boxed area is shown in **(H)**. **(H)** High magnification of the boxed area in **(G)**. The upper region of the nerve fascicle shows degenerative changes (eg, swollen axonal sheaths and myelin debris) (arrowheads). The lower region of the fascicle is essentially normal. A black line marks the approximate junction between the upper and lower regions. Mild lymphocytic inflammation is present in the connective tissue surrounding the nerve fascicle (left of photo, partly circled). **(I)** High magnification of the area indicated by an arrow in **(F)**. This field shows 2 longitudinal sections of nerve fascicles. Rows of digestion chambers, typical of Wallerian degeneration are indicated in the lower fascicle (arrows). This nerve bundle was not located in the immediate proximity of inflammation. **(J)** High magnification of the granuloma (marked G in **(G)**). The granuloma consists of a nodular cluster of giant multinucleated cells mixed with smaller mononuclear cells. Sample 70B: **(K)** low magnification. Severe inflammation surrounds a single large nerve bundle (outlined). Three collections of suture material are indicated (arrows). The boxed area is shown in **(L)**. **(L)** Medium magnification of the boxed area in **(K)**. Severe granulomatous inflammation and fibrosis surround the suture material (asterisk). Some suture material was partially lost in slide preparation. The granulomatous inflammation is arranged in sheets and discrete granulomas (arrows). **(M)** High magnification of granulomas with optically empty centers (asterisks), which are typical of those induced (eg, by CFA). A few giant multinucleated cells are indicated by arrows. **(N)** High magnification of 2 nerve fascicles with degenerative changes of the type shown above (in **(D)** and **(H)**). The surrounding connective tissue shows mild lymphocytic infiltration (asterisks). **(O)** High magnification of a nerve fascicle with mild degenerative changes and mild lymphocytic infiltration of the endoneurium (circled). Arrows highlight the perineurium. Inflammation within the endoneurium rather than in the connective tissue, external to the perineurium, was rare.

inflammatory mediators such as formalin, carrageenan, and CFA representing arthritis, and surgical nerve damage representing painful diabetic neuropathy and postherpetic neuralgia[32]; study design and reporting standards are inferior to those prevailing in clinical trials (ie, randomization and blinding methods); and it has been indicated that the level of stress involved in withdrawal testing in rodents might interfere with study measurements.[3]

The pig was therefore chosen for the present study. The pig's nerve morphology and functionality are similar to those of humans. For example, the average number of median nerve fascicles in the rat, pig, and human is 3, 34, and 37, respectively.[21,36,43] Unmyelinated afferents in pigs exhibit functional proportions of C-fibers in the skin that are similar to humans.[11,34] Structural changes observed in pigs' sciatic nerve when following chronic disease such as diabetes correspond to clinical findings in patients with hyperglycemia/diabetes-evoked peripheral neuropathy.[18] Relatively young animals were chosen for this study because pigs' responsiveness to mechanical threshold testing decreases as they gain weight.[16]

vF and feather stimuli were used to determine the reduction in the withdrawal threshold after PNT. These tests are also used in humans, among others.[23] The sensitivity of pigs to vF testing was in the range of human sensitivity after postoperative pain.[5] The feather test was used because it most closely resembles the light touch-evoked allodynia test in humans.

Unlike sciatic nerve injury models in rats, PNT did not result in significant changes in leg or foot position.[4] Changes in the animals' response to mechanical stimuli

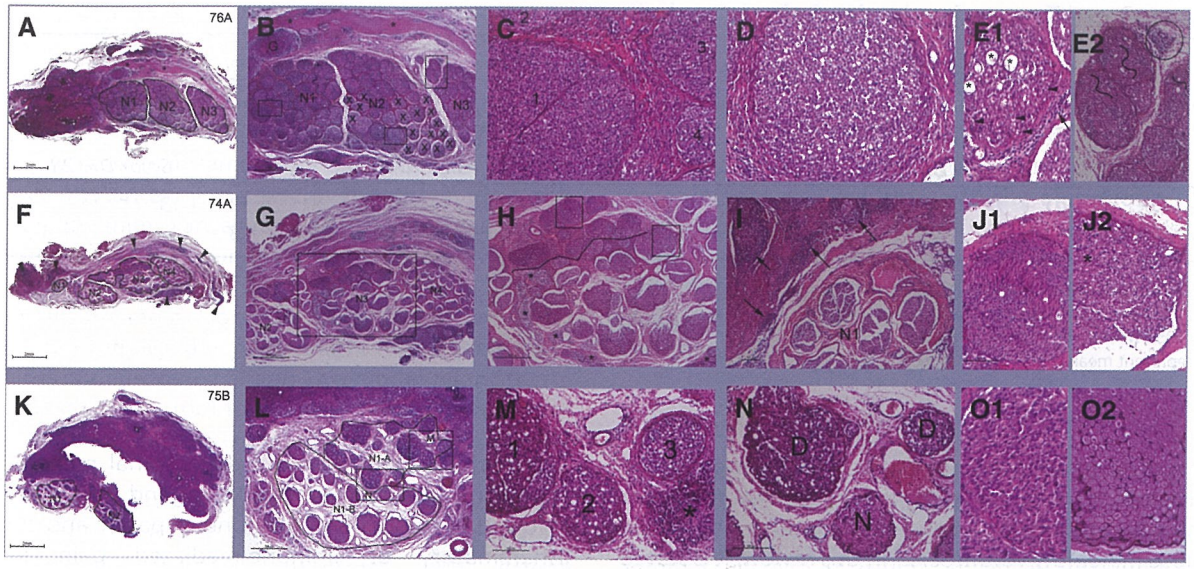

Figure 7. Histology samples from PNT pigs (study day 28). Sample 76A: **(A)** low magnification. Severe local, extensive granulomatous inflammation (left). Three nerve bundles (N1–N3) are right of field. Some inflammation is also present above a band of connective tissue fascia (asterisks). **(B)** Medium magnification of the central region. Degenerative changes are present in the 3 nerve bundles to varying degrees. Nerve fascicles with relative axonal preservation are marked X in N2. Nerve fascicles were also present in N3 but lie beyond the field included here. The boxed areas from left to right are shown in **(C)**, **(D)** and **(E2)**, respectively. The field shown in **(E1)** is not included in this image. It lies immediately outside the right border. G, granuloma; asterisk, fascia. **(C)** Medium magnification. This field shows N1 and includes the parts of 4 nerve fascicles (1–4). All fascicles show severe and potentially full axonal loss and SCHP. There is mild lymphocytic infiltration in the connective tissue, between the nerve fascicles. Minimal lymphocytic infiltration may also be present within the endoneurium. **(D)** High magnification. This field shows N2, a single nerve fascicle with widespread or possibly complete axonal loss and SCHP. **(E1)** High magnification. This field shows N3. Degenerative changes in the fascicle include marked swelling of a few axonal sheaths (asterisks) and mild SCHP. A few putative axons persist (arrowhead). There is lymphocytic inflammation in the connective tissue immediately outside the perineurium. **(E2)** High magnification. This field shows the upper part of N3. Nerve fascicles are cut obliquely and show the undulating pattern typical of SCHP (also referred to as Büngner bands). The black lines follow the wavy pattern of the hyperplastic Schwann cells. Sample 74 A: **(F)** low magnification. Mild multifocal inflammation and relatively limited degenerative changes in nerves. Arrowheads point to clusters of inflammatory cells. The most severe inflammation, which is moderate compared with most other samples in this study, is far left. Four nerve bundles are marked (N1–N4). **(G)** Medium magnification of the central region. Nerve bundles N2 and N4 are predominantly normal. N3 is partly normal and partly degenerate. The boxed area is shown at higher magnification in **(H)**. **(H)** Medium magnification of N3. Degenerate nerve fascicles are located above the black line crossing the bundle sideways. Left and right boxed areas are shown in **(J1)** and **(J2)**, respectively. Asterisks indicate small foci of mononuclear infiltration. **(I)** Medium magnification of N1. The nerve fascicles are normal. There is mild multifocal granulomatous inflammation in the adjacent connective tissue (arrows). **(J1)** High magnification of the boxed area on the left in **(H)**. The nerve fascicle shows typical SCHP/Büngner bands. **(J2)** High magnification of the boxed area on the right in **(H)**. This nerve fascicle is normal, with the possible exception of a small area where there may be mild SCHP (asterisk). Sample 75B: **(K)** low magnification. Local and extensive severe inflammation occupies most of this sample. Two nerve bundles are located at the lower edge (N1 and N2). The empty area running obliquely through the sample was probably where the suture material was located (it may have fallen out of the block or slide during histologic preparation). Nerve bundle N1 is partly degenerate. In N2, all nerve fascicles show severe degeneration. **(L)** Medium magnification of N1. Nerve fascicles located close to the inflammation (marked as N1-A) are all degenerate. Nerve fascicles located away from the inflammation (marked as N1-B) are normal. The boxed areas are shown in **(M)** and **(N)** as indicated. **(M)** Medium magnification. Three degenerate nerve fascicles are shown. There is a focus of mononuclear inflammation in the connective tissue located at the lower right corner (asterisk). **(N)** Medium magnification of N1 (from a field on the junction of N1-A and N1-B, as noted in **(L)**). Two degenerate (D) and 1 normal (N) nerve fascicles are indicated. **(O1)** High magnification of a nerve fascicle with diffuse axonal loss and SCHP. Compare with **(O2)**. **(O2)** High magnification of a normal nerve.

(vF or feather) were found during the entire study period. However, this study does not suggest the withdrawal response as a sole monitored parameter but also proposes an interpretation of detailed behaviors in pigs.

The subjective pain experienced by animals cannot be assessed.[53] However, the CBS proposed in this study[5,37] might help us understand the animals' persisting pain and the effect of drugs on this condition. In injured animals, an increase in CBS was observed on study day 7 and throughout the study period. The pigs showed a clear and coherent change in their behavior after PNT. Massive efforts are carried out to identify changes in behavior in rodents after pain. This difference might be because rodents are usually removed from their animal house and home cage and are moved to a different environment that is associated with pain testing.[46] This might lead to stress and perhaps to conditioning behavior that could affect their responses. In the present study, the animals were observed in their home pen. Another difference might be a result of the long interaction with 1 caretaker, who handled the animals from the first acclimation day and performed the observations. The caretaker may have learned the individual animal's behavior and could discern even the slightest change in its behavior.

The PNT model was challenged using standard pharmacotherapies: gabapentin (an anticonvulsant), morphine (an opioid), and aprepitant. All drugs were administered on study days 28 and 30 (ie, at the chronic

Table 5. Gene Expression in the Spinal Cord†

	CX3CR1 (Fold Change vs Control)				BDNF (Fold Change vs Control)			
	IL	CL	IL	CL	IL	CL	IL	CL
	Study Day 10		Study Day 28		Study Day 10		Study Day 28	
PNT animals	2.21 ± .28*,***	1.13 ± .06	1.00 ± .19	1.22 ± .15	.49 ± .12	.82 ± .31	5.53 ± 1.13*,**,***	.80 ± .30
SHAM animals	1.12 ± .08	1.04 ± .2	1.14 ± .16	.84 ± .07	.94 ± .08	1.18 ± .16	.98 ± .14	1.31 ± .05

*$P < .05$ ipsilateral (IL) vs contralateral (CL).
**$P < .05$ study day 28 vs study day 10.
***$P < .05$ PNT animals vs sham animals.
†Data represent mean fold vs naive animals (±SD).

neuropathic phase). The peak of activity after gabapentin treatment using the IV route was at 3 hours, similar to the maximum plasma concentration of a single 300-mg dose in healthy volunteers.[7] In this review, it is stated that in rats, gabapentin concentrates in the pancreas and kidneys. Pancreatic and renal tissue concentrations are 8 and 4 times higher than serum concentrations, respectively. In humans, the drug does not accumulate in the pancreas. A high similarity is suggested between the human and pig pancreas, both in function and in structure.[22] The similarity between the activity of gabapentin in humans and in pigs may be caused by a similarity in pancreatic function. Further pharmacokinetic study of gabapentin in pigs should be performed to test this assumption. Aprepitant, an NK-1 antagonist, was chosen because it has been studied extensively in rodent models, with promising results. However, it failed to show efficacy in the clinical testing phase. In this study, treatment with morphine or gabapentin had a reverse dose-related effect on vF and feather testing. Gabapentin and morphine at the highest dose tested completely reversed tactile allodynia. CBS, although reduced, was not completely abolished after the treatment. This difference between the effect of drugs on the responsiveness of animals to drugs using withdrawal assessment and behavior assessment should be further investigated.

We assessed the level of some common biomarkers that accompany chronic pain at the site of injury and spinal cord to further characterize this model at the molecular level.

Spinal cord biomarker analysis showed a significant but transient increase in the CX3CR1 level in the spinal cord on study day 10, suggesting involvement of microglia in the initiation of the pain. Spinal microglia[45,55] expressing CX3CR1 and the ligand (CX3CL1) are involved in the generation of neuropathic and chronic-inflammatory or immune-mediated pain in rodents[24,26,42,48,54] and in humans.[6]

BDNF increases in activated spinal cord microglia in the rat and mouse neuropathic pain models.[29,50] In BDNF knockout mice, there is a loss of mechanical hyperalgesia after nerve injury, suggesting that BDNF plays a major role in neuropathic pain.[51] In PNT-injured pigs, a 5-fold increase in spinal cord BDNF ipsilateral to the injured side was measured on study day 28. This increase in BDNF was negatively correlated with the decrease in CX3CR1, suggesting that the source of BDNF might be predominantly from nociceptors rather than from glia cells. Further studies are required to investigate this hypothesis.

BDNF also promotes neuronal growth, differentiation, survival, and maintenance of mature neurons.[41] The fact that BDNF expression in this study remained high on study day 28 suggests that the site of injury continues to release pain-related components. Similarly, a marked increase in CGRP mRNA level was found at the site of injury, peaking on study day 10. Rodent studies[12] showed that CGRP is a critical neuropeptide mediator for pain behaviors and upregulating innate immune responses. On study day 10, when BDNF and CGRP mRNA expression levels at the injury site peaked, TNF-α and IL-1β levels also peaked. IL-1β and TNF-α play a critical role in neuroinflammation and contribute to mechanisms of persistent neuropathic pain resulting from nerve injury.[1] Primary sensory neurons direct the effect of TNF-α on de novo synthesis of BDNF, and TNF-α-mediated increase in

Table 6. Gene Expression at the Site of Injury†

	TNF-α		IL-1β		BDNF		CGRP	
	Study Day 10	Study Day 28	Study Day 10	Study Day 28	Study Day 10	Study Day 28	Study Day 10	Study Day 28
PNT animals	5.03 ± 1.19	1.81 ± .34*	5.94 ± 1.59	1.91 ± .84*	193.47 ± 67.01**	127.75 ± 31.22**	10.36 ± 4.00**	4.07 ± .99**
SHAM animals	BDL	BDL	BDL	BDL	1.67 ± .95	1.99 ± 1.8	1.68 ± 1.99	.91 ± 1.2

Abbreviations: CGRP, calcitonin gene-related peptide; BDL, below detection level.
*$P < .05$ study day 28 vs study day 10.
**$P < .05$ PNT animals vs sham animals.
†Data represent mean fold vs naive animals (±SD).

BDNF expression is accompanied by an increase in CGRP.[1] On study day 28, IL-1β and TNF-α levels decreased.

Biomarker analysis of the spinal cord and site of injury suggest that this model might mimic 2 phases of pain: 1) an early increase in inflammatory components at the site of injury and an early increase in the glia marker; this phase might mimic an acute postnerve trauma phase; 2) a significant decrease in the inflammatory component at the site of injury with a significant increase in BDNF at the spinal cord and a strong presence of CGRP and BDNF at the site of injury; this phase might mimic the second chronic and neurogenic phase. This model might therefore also serve as a tool for further investigation of the shift between the acute and the chronic neurogenic phase.

Histologic findings suggest typical clinical manifestations of nerve trauma with frequent association of granulomatous inflammatory infiltrates and axonal degeneration.

The pig PNT model offers a new peripheral sciatic injury-induced chronic pain model. The model was developed in light of criticism of rodent pain models. The authors chose the pig because of the greater similarity between the nervous, inflammatory and cardiovascular systems of pigs and humans. This model relies on changes in the animals' behavior. A more clinically relevant test for light touch (feather) was added to the vF test that is commonly used in rodents. The proposed model may contribute to the elucidation of the mechanism underlying chronification of pain resulting from nerve injury. The study design involves standards reflecting clinical trials and blinding methods and was designed to minimize stressful conditions throughout the study period.

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