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RESEARCH ARTICLE

The refined biomimetic NeuroDigm GEL™ Model of neuropathic pain in the mature rat [version 1; peer review: 3 approved with reservations]

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Abstract

Background: Many humans suffering with chronic pain have no clinical evidence of a lesion or disease. They are managed with a morass of drugs and invasive procedures. Opiates usually become less effective over time. In many, their persistent pain occurs after the healing of a soft tissue injury. Current animal models of neuropathic pain typically create direct neural damage with open surgeries using ligatures, neurectomies, chemicals or other forms of deliberate trauma. However, we have observed clinically that after an injury in humans, the naturally occurring process of tissue repair can cause chronic neural pain.

Methods: We demonstrate how the refined biomimetic NeuroDigm GEL™ Model, in the mature male rat, gradually induces neuropathic pain behavior with a nonsurgical percutaneous implant of tissue-derived hydrogel in the musculo-fascial tunnel of the distal tibial nerve. Morphine, Celecoxib, Gabapentin and Duloxetine were each screened in the model three times each over 5 months after pain behaviors developed. A pilot study followed in which recombinant human erythropoietin was applied to the GEL neural procedure site.

Results: The GEL Model gradually developed neuropathic pain behavior lasting months. Morphine, initially effective, had less analgesia over time. Celecoxib produced no analgesia, while gabapentin and duloxetine at low doses had profound analgesia at all times tested. The injected erythropoietin markedly decreased bilateral pain behavior that had been present for over 4 months. Histology

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revealed a site of focal neural remodeling, with neural regeneration, as in human biopsies.

Conclusion: The refined NeuroDigm GEL™ Model induces localized neural remodeling resulting in robust neuropathic pain behavior. The analgesics responses in this model reflect known responses of humans with neuropathic pain. The targeted recombinant human erythropoietin appears to heal the ectopic focal neural site, as demonstrated by the extinguishing of neuropathic pain behavior present for over 4 months.

Keywords

animal models, neuropathic pain, erythropoietin, nerve regeneration, neuritis, tissue repair, hydrogel, nerve block, morphine resistance, refinement

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Introduction and background

The development of chronic neural pain following soft tissue injuries in humans is an uncommon but disabling complication^{1–5}. The persistent pain usually begins gradually, continuing for months to years. The typical initiating causes of the antecedent soft tissue injuries include blunt trauma, strains, surgery, industrial injuries, radiation, fractures, vibration, and repetitive motion^{6–16}. Disuse also contributes to the tissue matrix stiffness, edema and pain¹⁷. Despite a common history of trauma, a trauma-specific neural lesion or occult nerve injury are seldom recognized. The existence of an occult neural lesion has been implied in the initiation and the maintenance of neuropathic pain¹⁸. In the absence of an identified neural lesion, many of these patients have been hypothesized to have a peripheral neural “generator” or an “ectopic” site of localized inflammation^{19–30}.

The perceived absence of a specific neural injury site does not mean these patients lack such a lesion; these lesions may be “clinically invisible” and below our current level of detection³¹. These patients usually have no clinical evidence of either neural injury or physical abnormality^{18,32}. The persistence of pain behaviors in these individuals argues in support of a local neural activation site. *In vivo* peripheral nerve imaging techniques^{33–39} and diagnostics are presently being developed^{40,41}; however, they cannot yet detect abnormalities in small branches of the distal peripheral nerves¹⁸, which are the fibers most likely to be affected in soft tissue injuries.

A logical cause for the gradual appearance of chronic pain following soft tissue trauma is the predictable changes that occur during the tissue repair process at the affected site. These changes involve the removal of debris, fibrosis, and the regeneration of damaged tissue, including muscle, nerve, vasculature and extracellular matrix. The remodeling of tissue may result in nerve compression, with delayed onset of pain⁴². One such example of the ability of minimal pressure on the nerve causing severe pain, is trigeminal neuralgia, where even micro-compression of the nerve root can cause severe pain⁴³. The timing of the onset of chronic neuropathic pain parallels tissue morphologic events that occur during healing and tissue remodeling of the affected area (Figure S1: Tissue repair comparison chart)^{44,45}. We hypothesize that it is during tissue remodeling that an accumulation of matrix and possibly local edema alter the neural microenvironment⁴⁶ and contribute to the compression of vulnerable nerve cells, resulting in focal neural injury. These injuries cause atypical matrix forces then abnormal function⁴⁷ of peripheral glia and neurons⁴⁸, resulting in subsequent pain syndromes. To test this biophysical hypothesis we have created a model of a discrete focal lesion in the rat rear limb that recreates clinical findings found in humans.

Doubts have been raised about whether or not rodents can represent the human condition in neuropathic pain because few effective analgesics have been discovered using them^{1,49}. We consider the social behaviors⁵⁰, tissue healing^{51,52} and the similar evoked neural pain behaviors that humans share with rodents as confirmative to the relevance for their use. Two other crucial factors we used were the rodent’s correlated mature human age and the clinical relevance of the biologic pathophysiology embodied by the model^{1,53}. Presently, animal models with neuropathic pain behaviors are

created using forms of direct surgical nerve trauma or open surgery with neural irritation using chemicals, drugs, cold or heat^{54,55}. The most commonly used of these are the Spinal Nerve Ligation (SNL) model⁵⁶, the Chronic Constriction Injury (CCI) model⁵⁷, and the Spared Nerve Injury (SNI) model⁵⁸. These models use ligations, neurectomies or a combination to create pain with sensory and motor debility. While these open surgical models are useful in mimicking direct nerve trauma, they do not reproduce the pathophysiology of the delayed onset of neural pain without debility, as usually happens in many patients with neuropathic pain.

This biomimetic neural pain model is based on clinical observations of patients with soft tissue injuries followed by persistent pain. Many of them were treated with a specific localized nerve block for a regional pain, after a detailed neuroanatomic examination of their involved peripheral nerves (MRH physician practice 1987–2016). The NeuroDigm GEL™ Model uses a known physiological tissue process to create an occult chronic mononeuritis as seen in these patients.

Materials and methods

Ethical statement

The protocol was approved by the Institutional Animal Care and Use Committee of NeuroDigm Corporation (IACUC permit number 1-2014/15) and was in compliance with the guidelines of the 8th edition of Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used, and pain and suffering. NC3Rs ARRIVE guidelines for reporting on animal research were followed (Supplementary file S2).

Experimental animals

Thirty-seven Sprague Dawley 9.5-month-old male rats (an outbred strain from Harlan facility in Houston, Texas) were received, after being raised within their normal social groups. Their initial weights ranged from 440 to 660 grams, with a mean of 545 grams. In this study, the rats’ human equivalent age is as a mature adult^{59–61}. The rats had no prior drug exposure. A total of 37 rats were received with 36 rats enrolled after baseline testing. Three rats were removed from study for complications, with 33 finishing study.

Housing and husbandry

Ventilation and housing were in compliance with the guidelines of the 8th edition of Guide for the Care and Use of Laboratory Animals. Each rat was housed singly in clear, open cages in the same room. The room and individual cages had ammonia sensors (Pacific Sentry). No other animals or rodents were housed in the facility. The cages were changed out every 2 weeks or earlier. Bedding was 0.25-inch corn cob pellets. Food was LabDiet 5V5R with low phytoestrogens, continuous access. Light-dark cycle was 12 hours, with lights off from 7 PM to 7 AM, except when screening. Maximum lumens at cage level were 20–40; at time of pain behavior testing the maximum lumens were 85–100. The room had no high frequency interference detected (Batseeker Ultrasonic Bat Detector), other than that related to the rats on weekly and as needed testing. Water used was municipal water. In each cage, enrichments were 1) a non-plasticized polyvinyl chloride tube 4” in diameter by 6” in length (Bisphenol A free) for shelter and 2) bedding at an increased depth of 0.75 to 1 inch when dry, to

encourage burrowing. Facility was in north Texas. All pain behavior testing were performed in the same room as the rats were housed.

Study design

After receiving, the rats were acclimated for 15 days with baseline testing (Figure 1). The rats were housed singly to limit fighting and rough play. The rats were randomly assigned to one of three groups: GEL procedure, sham procedure, or control (no procedure). The investigator performing the procedures and behavioral testing was blinded to the rat group assignment. Another experimenter did the random group assignment, before the initial procedures. The rats were housed in a separate room during the procedures and handed to the investigator by an assistant. Tail identification was masked prior to performance of the procedures. The investigator did not know the group assignments at any time, until the unblinding on post procedure day 149. The locations of the animals on the rack were randomly changed every 10–14 days. Isoflurane 2–3% was used for anesthetic induction and during the procedure for approximately 2 minutes. Isoflurane gas was used due to brief anesthetic time needed, enabling less recovery time compared to most injectable anesthetics. Four analgesics, morphine, celecoxib, gabapentin and duloxetine, were screened three times each during the 5-month study. The screening involved testing the plantar hindpaws of the rats with stimuli used to detect mechanical allodynia and mechanical hyperalgesia. Each rat was tested singly on a wire mesh with manual application of stimuli, for all behavior testing. The behavioral testing was performed between 1 PM and 11 PM. Animal welfare observations of behavior, coat and movement were checked daily. Monitoring for signs of infection, water and food use were conducted at least three times a week. Weights were monitored every 4 weeks, or every 2 weeks as indicated. The same female investigator performed all procedures and screenings, with no one else in the room. Subcutaneous injections rather than oral gavage were used for analgesics, as they are less stressful. A pilot study, blinded during pain behavior testing, was performed on the effect of a localized application of a human recombinant erythropoietin analog (EPO) injected near the GEL™ neural procedure site on post procedure day 152, followed by behavioral testing on days 153–160.

Sample size

We tested the null hypothesis⁶² that the GEL procedure does not differ from the control group during the 5 months after procedure on the dependent variables of paw withdrawals in response to von Frey fibers, a camel-hair brush, and pinprick. The alternative hypothesis was that, over time, there is a difference between the groups. The experiment was designed to discover the smallest biologically important effect, optimizing the number of animals used⁶³.

We conducted a power analysis based on data from previous rat experiments with the GEL model to detect a difference between the GEL group and control group with an unpaired *t*-test if the difference was 1 paw withdrawal and the standard deviation was 0.5 paw withdrawals. We concluded that a minimum sample size of eight per group would yield 95% power with a two-tailed Type I error rate of .05. An additional three animals per group were added to compensate for a possible loss of sample size during the 5-month study, and an additional four animals were added to the GEL group for illness over time, technical complications, and a pilot study with local EPO. GEL *n* = 14, control *n* = 11, sham *n* = 8 were the final sample sizes.

Experimental procedures

Percutaneous injectable procedure for GEL and sham. During isoflurane anesthesia the percutaneous injection procedures were performed. The hydrogel used in the GEL group was the proprietary biological NeuroDigm GEL™, that is composed of purified biocompatible tissue-derived by-products and peptides of mammalian soft tissue, as found in the perineural tissue milieu after a soft tissue injury. Such injectable implant products are used in human surgeries, dermal procedures, and wound healing, with rare reactions of acute inflammation. Purified biological proteins and hydrogels are normally absorbed over days to weeks by the tissues into which they are implanted, and are rarely antigenic^{64,65}. The hydrogel we used was introduced within the left (ipsilateral) tibial neural tunnel below the popliteal area at mid lower leg, with aseptic technique. First, the skin was pierced with a sterile 19 gauge

NeuroDigm GEL™ Model Study Timeline

Figure 1. Timeline: Three groups had four drugs screened for analgesia three times over the 5-month study. At the end, a pilot study with localized EPO was performed.

needle tip; then a sterile, custom tapered, blunted 21 gauge hollow probe entered the skin puncture site to gain access to the tibial nerve tunnel (U.S. patents 7015371, 7388124). Since older confined rats normally have less passive knee extension, a distal to proximal neural tunnel access was used in this study. The point of the probe's percutaneous entry was over the Achilles tendon. The probe then advanced subcutaneously in a caudal direction; then it pierced the fascia between the distal origins of the medial and lateral gastrocnemius muscle and entered the anatomic tunnel posterior to the tibialis posterior muscle and medial to the soleus, where the tibial nerve courses. Upon entering the neural tunnel, the probe was softly glided in avoiding resistance or nerve contact. In the mid-tibial tunnel of the lower leg 0.3 cc of the GEL™ or the shams' Ringer's lactate was deposited, and then the probe was withdrawn, with the rat placed in a cage for observation.

Procedures to elicit neuropathic pain behavior. The primary outcome measure was the average number of paw withdrawals to each of five stimuli applied eight times to each plantar mid hindpaw of a rat. Each stimulus was applied first to the contralateral hindpaw, then to the ipsilateral side of the GEL and sham procedure. Time between each stimulus application was usually 2–4 seconds or longer. For each stimulus the total number of each hindpaw withdrawals was recorded as a data point.

Measures of mechanical hypersensitivity were chosen for this analgesic study. These measures are the most commonly used in rodent screening for analgesics and also commonly used in humans. Further behavioral characterization of this model is planned.

Non-noxious light touch: for static mechanical allodynia von Frey filaments (Semmes Weinstein Mono-filaments North Coast Medical TouchTest®) exerting confirmed forces of 2 grams, 6 grams and 10 grams were used, tips smooth. Dynamic mechanical allodynia was tested with a fan sable brush (09004-1002; Dick Blick Art Materials). The stimuli were applied in the order of: von Frey 2 g, 6 g, 10 g, then brush. Each von Frey stimulus was applied for approximately 1 second until the fiber bent, or the paw was withdrawn. The brush was stroked gently from rear to front of the plantar hindpaw.

Noxious light touch: mechanical hyperalgesia was tested with a custom sharp non-penetrating plastic point calibrated to elicit 2–4 paw withdrawals at baseline. This pinprick stimulus tip was touched to the plantar site until the paw withdrew or the skin visibly indented. Each stimulus lasted about 1 second.

Analgesics administration. The analgesics were administered by subcutaneous injection (27 g 1.5”) over the dorsal lower back and proximal thighs, with a custom administrator-held restraint device to reduce handling and stress. Morphine sulfate (West-Ward) was mixed with normal saline and administered at a dose of 3 mg/kg 1 hour prior to screening the analgesic. The vehicle used in mixing the following three drugs was 0.25% methylcellulose (Methocel® A4MPremium LV USP). These three drugs were mixed 24–48 hours prior to use. Celecoxib (Cayman Chemical) was dispensed at a dose of 10 mg/kg 1 hour prior to screening; gabapentin (Cayman Chemical) was dispensed at a dose of 25 mg/kg 2 hours

prior to screening; and duloxetine (Cayman Chemical) was mixed (mechanically agitated) and administered at a dose of 10 mg/kg 2 hours prior to screening. The experimenter knew the drugs being screened; the identity of the groups were blinded throughout experiment. The injected volume of each drug was less than 1.2 cc.

The original doses chosen for gabapentin and duloxetine had adverse effects in this study of aged mature rats, interfering with the testing of pain behaviors. Gabapentin at 60 mg/kg had marked ataxia in all rats, with their hindpaws not staying on testing screen due to lumbering gait and falls. Duloxetine at 30 mg/kg was noted for marked “frozen” hypoactive posture, with increased tone and alertness (no central sedation) to normal handling and testing. After duloxetine was given at this dose, paw withdrawals were not elicited in any of the three groups. Due to these adverse effects, lower doses were tested and used, as described above. These lower doses had no observed drug side effects and improved the ability to test paw withdrawals⁶⁶.

Erythropoietin treatment pilot study

Epoetin alfa (EPO) by Amgen, a recombinant human erythropoietin analog at 2000 units/mL, was diluted 1:3 with normal saline, and 0.3 cc vol., 200 units, was the administered dose in the pilot study. After the main experiment was over the 14 GEL procedure rats continued in a pilot study for 8 days beginning on day 152, with days 140, 149 and 152 taken as baseline days. Three subgroups were picked randomly and the experimenter was blinded during screenings for pain behavior. On day 152 under isoflurane, as described prior, the “EPO at site” group ($n = 5$) received an injection of 200 units of EPO at the site of the original GEL procedure on the ipsilateral leg. The “EPO SC” group ($n = 4$) received the same EPO injection subcutaneously by dorsal low back, and the “No EPO” group ($n = 5$) received no injection. The original “EPO at site” injection approach was ipsilateral (left) posterior-to-anterior at mid tibia through the bellies of the gastrocnemius muscle aiming for the tibial nerve tunnel. Pinprick behavior data were collected on days 153, 154, 156, 159, and 160.

Two of the five “EPO at site” rats had no decrease in paw withdrawals with the original technique of the EPO injection. These two rats had an adapted lateral approach of the injection of 200 units of EPO to improve localization of the perineural infiltrate near the original ipsilateral GEL™ procedure site. This adapted injection was on the ipsilateral side, through the lateral gastrocnemius muscle targeted to the mid tibial tunnel at lower leg.

Histology

At the conclusion of the study, three rats were chosen randomly from each of the three groups: 1.) GEL procedure rats 2.) sham procedure rats of the 5/8 that displayed late onset robust pain behavior, and 3.) controls. The selection from the GEL group contained two rats that were controls in the EPO pilot study, and one that had received the subcutaneous EPO injection, all with no change in pain behavior noted. The animals were anesthetized, and then perfused with Lactated Ringer's solution (Hospira), followed by perfusion fixation with 4% paraformaldehyde (PCCA Professional Compounding Centers of America) in Phosphate Buffered Saline

(PBS) (Electron Microscopy Services). Following fixation, the lower limb on the ipsilateral side was grossly dissected to reveal the gastrocnemius muscle thus providing a landmark for locating the tibial nerve. Once identified, the distal tibial nerve (below the popliteal area) was dissected free of the surrounding muscle and fascia, and placed into ice-cold 4% paraformaldehyde in PBS for overnight incubation. The following day the paraformaldehyde solution was replaced with 30% sucrose (IBI Scientific) to cryoprotect the tissue. The cryoprotected samples were embedded in Tissue-Tek OCT (Sakura Finetech, Japan) and frozen on dry ice. Cryosections (10 μ m) were then prepared and mounted onto SuperFrost Plus slides (Fisher Scientific, Rockford, IL). Sections were then fixed in 10% Neutral Buffered Formalin for 10 minutes, washed for 5 minutes in 1X PBS to remove OCT, and rinsed with tap H₂O. Subsequently, sections were then stained in Hematoxylin (Fisher Scientific) for 5 minutes and rinsed with tap H₂O, differentiated in acid alcohol (1% HCl in 70% EtOH) for 30 seconds and rinsed extensively with tap H₂O, blued in 0.2% aqueous ammonia, rinsed with tap H₂O, and stained with eosin (Fisher Scientific) for 1 minute. Sections were then dehydrated by sequential submersion in graded 75%, 95%, 100% EtOH for 5 minutes each, and a final submersion in xylene. The slides were air dried before mounting with Permount (Fisher Scientific) and adding coverslip. Sections were viewed and the images captured on a Nikon 80i microscope, outfitted for digital light micrographs.

Statistical methods

Pain behavior statistical analyses. As described in detail in the results section, the data were inspected for compliance with the assumptions of ANOVA. Two areas of concern were noted, particularly the heterogeneous variances in the pinprick data and the very large number of pairwise comparisons that could be compared. The former occurred because (a.) GEL animals that developed pain symptoms tended to score a maximum number of withdrawal responses (8) out of the possible number of stimuli presented (8) leading to some cells with very small or zero variance in the GEL group only, and (b.) the animals in the sham group were not homogeneous in their response to the sham procedure and this greatly increased their variance. We proceeded with the ANOVA for pinprick because of the convenience of describing interaction effects and for comparison with the allodynia data. We note that the pinprick variable was the least likely to generate errors of inference because of the very large effects and consequent minuscule *p* values obtained. Individual sham data are plotted in a separate graph to illustrate the problem there. Type I errors were reduced by testing only planned comparisons among a relatively small number of means and by combining data where appropriate before analysis so that fewer comparisons would be made.

Analyses of the paw withdrawals in response to von Frey fibers, the brush, or the pinprick on the routine test days were conducted using a mixed model ANOVA with one between groups factor (eleven controls, fourteen GELs, and eight shams) and two repeated measures factors. The first repeated measures factor was the time the data were collected, with an average baseline period of 4 days prior to the procedures and the five 30-day periods, referred to as post procedure Monthly Periods 1 through 5 (P1, P2, P3, P4, P5), following the procedure (Figure 1). For analyses, the data point

for each animal in each monthly period was the mean of at least four routine pain behavior testing days during that month. The second repeated measures factor for the von Frey fiber analysis was a composite factor combining the three levels of fiber force (V1, V2, V3) and the two sides for a total of six levels of different forces tested on bilateral hindpaws. Differences owing to fiber force and sidedness were determined by comparing means with planned comparisons. The second repeated measures factor for the brush and pinprick were the bilateral hindpaws. In the global ANOVA, a *p* value of <.05 was considered significant. Except for the von Frey analysis, planned comparisons were conducted using Fisher's Least Significant Difference test after a global ANOVA was determined to be significant at the .05 level with a two-tailed test (Dataset was used in all analyses).

Analgesic statistical analyses. Experiments were conducted with four analgesic drugs administered shortly before the usual testing with von Frey fibers, the brush, and the pinprick. The four drugs were each tested three times during the post procedure period from day 28 to day 149. The effects of the analgesic drugs were analyzed on two dependent variables instead of five (allodynia measures averaged together as one variable and the hyperalgesia measure of pinprick as the other). The data were analyzed using a mixed model ANOVA with the three groups as a between-subjects factor and side (left or right) and days (three pairs of pre-drug and analgesic drug days) as repeated measures factors. The effect of the analgesic drug for each pair of days was analyzed using planned comparisons. These comparisons used Fisher's Protected Least Significant Difference test if the corresponding *F*-ratio was significant or used a Bonferroni-protected contrast if the *F*-ratio was not significant. All tests used a two-tailed significance level of .05.

Results

Behavioral observations

All rats had recovered from anesthesia within 5 minutes and were walking normally without altered gait. Following recovery from anesthesia the subjects did not demonstrate observable pain behaviors⁶⁷ nor clinical evidence of tissue injury. Throughout the duration of the study all the rats were observed to have normal gait and were without visible evidence of inflammation, swelling, weakness, deformities or positional changes noted on the operated hindpaw, at any time. There was no observed evidence of acute nociceptive pain, even after the procedures. Their grooming activities were normal. Among the GEL group, 14/14 rats had markedly increased paw withdrawals to pinprick, von Frey fibers and brush by day 23 post procedure; the paw withdrawals to pinprick became more exaggerated over the remaining months. By post procedure day 60, 5/8 of the sham rats had developed marked paw withdrawals. The most common pain behavior paw withdrawal reaction was a reflexive flinch. Other reactions appearing 1 month after increased pain behaviors included prolonged shaking and or licking of their affected ipsilateral paw. Similar patterns of paw withdrawal reactions occurred on testing the contralateral side as pain behavior appeared. No chewing of the paws occurred.

Results are presented below with the statistics; also see [Supplementary file S3: Basic Anova for a comprehensive](#)

description of the pain behavior statistical results without drugs or EPO.

Results of behavioral data

Pain behavior analyses. While the hyperalgesia in this mature GEL™ model was robust over time; the allodynia was a minor response. Before attempting a statistical analysis, we plotted the raw routine days of data (without analgesics) of each group for inspection. The robust effects of pinprick hyperalgesia were evident in the raw data graph. The small effects observed with the data of each individual allodynia stimulus (each of three von Frey fibers and the brush) indicated that an individual routine days mean in the GEL group could not reliably be expected to differ from the control group. In order to observe more reliable differences, the pain behavior data could be grouped for more samples in two ways.

First, *different days* of testing for each stimulus could be averaged together for an individual variable, such as data for each individual von Frey fiber averaged together over 4 testing days to make a monthly average: This method was used in the allodynia and hyperalgesia line graphs (Figure 3–Figure 5).

Second, the scores for *all four allodynia measurements* could be averaged together to make a summary single variable, i.e., averaging data for all three von Frey fibers and the brush into a single number representing allodynia. This method was used for allodynia in the analgesic drug response bar graphs, to compare one day of pre-drug data to one day of post-drug data (Figure 7–Figure 10).

These methods have different advantages. Plotting each day's data is useful for determining the precise timing of when effects emerge during the long period of testing. Plotting monthly data is useful for observing the small effects that are apparent between the individual von Frey fibers. Information about effect sizes will be presented for the routine days data of the averaged variable (von Frey plus brush), and formal statistical analysis will be applied to the much smaller number of means in the monthly data for each individual variable.

Days of data pattern of allodynia and hyperalgesia after GEL procedure. The data for all routine testing days (no analgesics given) for the combined allodynia variable (von Frey plus brush, top) and for the hyperalgesia variable (pinprick, bottom) are presented in Figure 2 below. The most noticeable effect is the increase of pain

Figure 2. Test days without analgesics; average of all allodynia responses (top) and average of pinprick responses (bottom). The averaged allodynia measures on the ipsilateral side were more consistently different on a routine test day basis than any of the individual allodynia measures (top: y-axis is 2, maximum is 8). Allodynia effects on the contralateral side are not obvious. Effects related to pinprick hyperalgesia were more robust and persistent than those for the allodynia measures (bottom: maximum y-axis is 8). Shams are not homogenous: 5/8 with pinprick hyperalgesia, 3/8 similar to controls.

behaviors in response to the pinprick in the GEL group on both the ipsilateral and contralateral sides. The pinprick hyperalgesia effect occurred in every rat subjected to the GEL procedure. Although the pinprick responses required about 23 days to develop, the symptoms, and therefore the opportunity to study those symptoms, persisted for months and showed no sign of waning by the end of the experiment. Under conditions of a null effect, each of two groups would be expected to have a greater mean than the other about 50% of the time. However, the last day on which the control group had an absolutely greater mean was for pinprick post procedure day 5 on the ipsilateral side and day 23 on the contralateral side.

Table 1 provides estimates of effect sizes for the GEL™ effect, which tends to increase with time. The standardized effect size was calculated as the difference between the means of the GEL and control groups on that day divided by the pooled standard deviation of the two groups. These can be used to plan future experiments depending on which interval after the procedure will be studied. Minimum sample sizes are provided to yield at least 80% power in a two-group, two-sided *t*-test with a Type I error rate of .05. Larger sample sizes are required to study allodynia than to study hyperalgesia. More complex designs, such as this one, which include many repeated measurements and multiple groups, will have more error degrees of freedom for the comparisons than a simple *t*-test, and will not require such large sample sizes for the allodynia measures.

Using these data, analgesic screening can start on day 23 or later with a group of $n = 6$ rats using pinprick (mechanical hyperalgesia) on the ipsilateral side. The effect size for hyperalgesia on the ipsilateral side was persistent at more than 1.94 standard deviations from day 23 until the end of the main experiment on day 149. Smaller effects of the GEL group were observed for the combined allodynia variable (von Frey plus brush) than for hyperalgesia with the pinprick, but, unlike the data for the individual von Frey and brush variables, the combined variable showed clear and persistent differences between the GEL group and control group on the ipsilateral side for each testing day. This is important for our subsequent experiments with analgesics. The effect of the GEL™ procedure is not constant across time. Therefore, when testing the effect of an analgesic on a single day with a control value from the same animal, the response must be compared to a control day very close in time to the day the analgesic is given rather than to the average of all control days. To do this successfully, each control day must show a positive effect of the GEL procedure, and this was not true on every day for the individual von Frey fibers or the brush alone. Consequently, we opted to use the combined von Frey plus brush data for all comparisons between individual analgesic and control days (see the section, *Results of experiments with analgesic drugs*, where statistical analyses of those days are provided).

In order to provide a formal statistical analysis of the individual allodynia variables, we averaged all days (at least 4) within each

Table 1. Effect size (ES) data for hyperalgesia and allodynia on routine test days for control (C) and GEL (G) groups.

Effect	Ipsilateral	Contralateral	Calculated n
Hyperalgesia (Pinprick)			
Last day C > G	5	23	NA
ES > 1.94 SDs	day 23–149	day 72–149	6
ES > 2.83 SDs	day 46–149	day 92–149	4
ES > 3.50 SDs	day 72–149	day 106–149	3
Allodynia (von Frey plus brush)			
Last day C > G	19	149	NA
ES > 0.71 SDs	day 23–149	--	32
ES > 0.86 SDs	day 112–149	--	22
ES > 0.91 SDs	day 119–149	--	20

Included are the last day that the control group mean exceeded the GEL group mean, and the inclusive days that the size of effect for the GEL group exceeded the control group by 1.94, 2.83, or 3.50 standard deviations (SDs). ES is the difference between the group means divided by the pooled standard deviation of the two groups. The calculated n is the sample size required to detect a difference between GEL and control groups of the given size for the hyperalgesia or allodynia variable on a routine test day basis in an independent-samples *t*-test with 80% power and a two-sided alpha of .05. Allodynia was not consistent on the contralateral side. See routine test day means in [Figure 2](#). NA, not applicable.

Figure 3. Mechanical allodynia to light touch over 5 months. Increased pain behavior of allodynia to light touch with von Frey fibers only seen in the GEL rats, bilaterally; on the left with 2 g, 6 g and 10 g fibers, and on the right with 6 g and 10 g. This allodynia decreased after the 3rd month for all fibers with this effect. These graphs depict von Frey fiber results for paw withdrawals ipsilateral and contralateral, for all groups, for three fiber forces over 5 monthly post procedure periods (P1–P5) (maximum y-axis is 8). Mean and S.E.M. * $p < .05$ GEL group greater than both GEL group baseline and control group for same period. Reduced responding in the GEL group during period 5 may reflect habituation.

month that the animals were tested without analgesics to remove some of the test day variability, stabilize the means, and estimate effect sizes. These analyses are presented in the following sections.

Mechanical allodynia: von Frey analysis. The data are given in Figure 3 for the three different fiber forces in each group, over all periods applied to both the ipsilateral and contralateral sides. The highest-order interaction of the ANOVA was significant ($F(50, 750) = 2.21, p < .001$). The control group never significantly exceeded the baseline value in any monthly period on either side. Asterisks in Figure 3 mark means of the GEL group that were significantly different from both the GEL group baseline and the mean of the control group during the same period. The shams had no significant allodynia.

Mechanical allodynia: brush analysis. The GEL group showed prolonged pain behavior of dynamic mechanical allodynia to brush stimuli with increased paw withdrawals, only on the ipsilateral side. This pain behavior peaked by the 3rd month then waned, returning to near baseline by the 5th month. The shams had similar pain behavior that plateaued by the 4th month, persisting until the 5th month at end of study. The response of the shams, with the onset of the pain behavior of mechanical allodynia during the 3rd month that persisted, was not anticipated (Figure 4). The highest-order interaction was significant ($F(10, 150) = 1.943, p = .044$). The control group never significantly exceeded the baseline value in any monthly period on either side. Asterisks in Figure 4 mark means of the GEL group that were significantly

Figure 4. Dynamic mechanical allodynia to brush over 5 months. Dynamic allodynia or hypersensitivity to the brush was noted only on the ipsilateral hindpaw. These graphs depict brush results for paw withdrawals ipsilateral and contralateral to procedure over 5 monthly post procedure periods (P1–P5) (maximum y-axis is 8). Mean and S.E.M. * $p < .05$ GEL group greater than both GEL group baseline and control group for same period. Reduced responding on the ipsilateral side in the GEL and control groups during period 5 may reflect habituation, which is not present in the shams as their allodynia increased in P4–P5.

different from both the GEL group baseline and the mean of the control group during the same period.

Mechanical hyperalgesia: pinprick analysis. The GEL group had the earliest and most persistent pain behavior of mechanical hyperalgesia with increased paw withdrawals to pinprick, bilaterally. The hyperalgesia was first present on the left side and within a few weeks present on the right side. This pain behavior was vigorous after the first month and persisted robustly for 4 months, until the end of the study. The shams had similar pinprick pain behavior bilaterally that peaked by the 4th month and persisted until the 5th month, at end of study. The control group had no pinprick pain behavior during the study.

The data for pinprick are presented in Figure 5. The highest-order interaction was significant ($F(10, 150) = 4.592, p < .001$). The control group never deviated from its own baseline value in any post procedure period on either side. By contrast, the GEL group's paw withdrawal response on the ipsilateral side was significantly greater than baseline during all five post procedure periods, and the GEL group on the contralateral side was significantly greater than baseline during periods 2 through 5. Between-group comparisons for pinprick indicated that the three groups were not significantly different during the baseline period on either the contralateral or ipsilateral side. Asterisks in Figure 5 mark means of the GEL group that were significantly different from both the GEL group baseline and the mean of the control group during the same period.

Figure 5. Mechanical hyperalgesia to pinprick over 5 months. The GEL and later the sham groups developed mechanical hyperalgesia to pinprick bilaterally. In the GEL group, 2–3 weeks following the onset of hyperalgesia on the left it began to develop on the contralateral side; a similar slight delay in the contralateral onset of hyperalgesia is seen in the shams. The graphs depict the mean of all paw withdrawal responses to pinprick on the ipsilateral and contralateral sides during the 5 monthly post procedure periods (maximum y-axis is 8). Shams are not homogenous: 5/8 with pinprick hyperalgesia, 3/8 similar to controls. No habituation effect was noted.

Individual data for sham group. Retrospectively, we noted that five of eight sham procedure animals developed pain behavior bilaterally, similar to the GEL™ animals, in post procedure monthly periods 4 and 5 (after 3 months); and the three remaining sham rats behaved similarly to the control group.

The foregoing analysis did not stress any effects that might or might not be different between the GEL and sham groups. The reason for this is that the sham group itself was not homogeneous in the responses of the animals to the sham procedure. Individual data for the sham animals are presented in Figure 6. The data on the shams, in all the pain behavioral studies and in the analgesic screening, included the results from all eight shams. The probability that the bracketed five responder sham rats would separate themselves from the other three in exactly the same direction by chance in the allodynia experiment is .017 for a single day. This probability does not factor in the magnitude of the effect between responders and non-responders or the fact that they separated themselves the same way on the same two consecutive days as in the hyperalgesia experiment. This is very strong evidence that we detected ipsilateral allodynia in all of the same sham animals where we detected hyperalgesia.

Summary of pain behaviors. For the GEL procedure group, we found that the gradual pattern of development of ipsilateral pain behaviors was usually followed with the gradual onset of contralateral pain behaviors within 2–3 weeks. In the GEL group, the light touch mechanical allodynia responses had a gradual onset after the first month, decreasing slowly after the third month (P3). Once the

pinprick hyperalgesia developed in the GEL group it became robust and persisted until the end of the study.

The sham procedure also gradually induced the pain behavior of pinprick similar to the GEL procedure in five of eight rats on the ipsilateral side after day 72, followed by the contralateral side after day 90. The affected shams (5/8) with elicited pain behavior of mechanical hyperalgesia became robust after the third month (P3), persisting until the end of the study. The shams did not have a robust response to von Frey or brush stimulation. The control group developed no pain behaviors.

Factor influencing pain behavior testing. A reportable factor discovered during this study relates to the influence of hormone replacement of the experimenter on the elicited responses to stimuli. On each routine pain behavior test day, after the first six random rats were screened, the results were compared to the prior session of each rat, to observe for environmental influences. In nine such early screening comparisons, paw withdrawals were markedly less to nonexistent bilaterally in the six rats prescreened as compared to their prior screening session. Thirty minutes after the topical application to the investigator of a 17 beta-estradiol replacement cream, the screening was repeated on the same six rats, and their elicited pain behaviors were then consistent with the data collected during the prior testing period. On unblinding, this effect was not related to groups. Even baseline pre-procedure behaviors were similarly affected by the estrogen hormone replacement. All data used in this study was collected with topical estrogen applied 30 minutes prior to beginning all testing. This reaction echoes the olfactory ‘male observer’ effect of male experimenters reducing acute pain behaviors in rodents, as compared to females⁶⁸; and suggests that besides their sex, the age and hormonal status of experimenters may influence the reproducibility of pain behaviors.

Figure 6. Individual sham data over 5 months. Graphs depict average of paw withdrawals for eight individual shams; the average of all responses to von Frey and brush stimuli (top) or to pinprick (bottom) over baseline and for 5 months (maximum on y-axis is 8). Pain behavior in response to pinprick on right developed 2–3 weeks after the left; 5/8 shams had bilateral pinprick mechanical hyperalgesia during the P3 month persisting until end of study. The remaining three animals resembled the control group. Bracket in allodynia graph identifies the same five responders from the hyperalgesia data. In P4 and P5 for bilateral pinprick there is complete separation between the three behaving like controls and the five behaving like GEL.

Results of experiments with analgesic drugs

To control for the effects of time, it was important to compare the data for each analgesic's screening day to a single pre-drug control day, prior to the drug's administration. As illustrated in the top part of Figure 2, an effect on the GEL group's ipsilateral side was apparent on individual control days when the paw withdrawals for all four allodynia measures were averaged together into one variable. For that reason, we analyzed only the composite allodynia variable and the pinprick for responses to the analgesics. The data for each analgesic drug are displayed in the figures as bars for the mean response, with the analgesic drug on a particular day (the days listed in the x-axis label) paired with the respective control data from the routine test day one to four days prior. Throughout the following analyses, the mean number of paw withdrawals in the GEL group on the ipsilateral side was significantly greater than that of the control group on all pre-drug days for both the von Frey plus brush variable and for the pinprick.

Morphine. Morphine sulfate at 3 mg/kg had an escalating loss of effectiveness bilaterally, which was not due to tolerance as each of the three doses were at least 30 days apart. Morphine at 3 mg/kg is usually a toxic dose in humans. The data for the morphine test days using the von Frey fibers and brush are presented in the top half of Figure 7. The ANOVA revealed that all main or interaction effects were significant at the .05 level including the three-way interaction

Figure 7. Morphine analgesia results. Morphine was less effective over time, not due to tolerance, since each dose is separated by weeks. The graph depicts the average of paw withdrawals on pre-drug control days (black) and on paired morphine dose days (white). Results of behavior testing show the average of all four light touch allodynia measures (three von Frey fibers and the brush, top graphs) and the mechanical hyperalgesia (pinprick, bottom graphs). Only on D28 morphine had marked analgesia with pinprick (bottom) and light touch allodynia (top) across all groups, bilaterally. The data suggest a developing opioid related hypersensitivity to stimuli. Mean and S.E.M. *Significant decrease from the paired control day, $p < .05$. + Significant increase from the paired control day, $p < .005$.

($F(10, 150) = 2.322$, $p = .014$). Morphine caused significant decreases in responding compared with pre-drug day on both the ipsilateral and contralateral side only on day 28 as denoted by asterisks in the figures. In three instances, morphine actually increased pain responses, as denoted by red plus symbols. These were the only significant increases in pain behavior resulting after an analgesic in the entire dataset for the four analgesic drugs. The data for pinprick are presented in the bottom half of Figure 7. The ANOVA revealed that all main and interaction effects were significant including the three-way interaction ($F(10, 150) = 2.655$, $p = .005$). Significant decreases in paw withdrawals are denoted by asterisks.

Morphine was effective early after the GEL™ procedure, but the size of the effect waned with time on both sides. For example, in the GEL group on the ipsilateral side for the allodynia measures, the standardized effect size between the control day and the morphine day changed from a positive analgesic effect of 1.30 pooled standard deviation units on day 28 to no effect on day 64 to a negative effect of -1.25 pooled standard deviation units on day 108. For the pinprick measure, effect sizes were conservatively estimated using the standard deviation for the morphine condition only instead of the pooled standard deviation because of the reduction of the variability as the responses approached a ceiling of eight paw withdrawals out of eight pinpricks. The analgesic effect size waned from 4.14 standard deviations on day 28 to 0.64 standard

deviations on day 108. The shams and controls also suggest an escalating pattern of stimuli sensitivity after morphine with pinprick.

Celecoxib. There was no analgesic effect of Celecoxib at 10 mg/kg in any group on any day on either side. Celecoxib did not decrease pain behaviors, as demonstrated with a decrease in paw withdrawals. Celecoxib at 10 mg/kg is about three times a human dose. Data for the celecoxib days for the von Frey fibers and brush are presented in the top half of Figure 8. The ANOVA revealed significant main effects of groups, side, and drug response and a significant group-by-side interaction ($F(2, 30) = 22.92$, $p = .001$). None of the other interaction effects was significant. There was no effect of the celecoxib dose on either side in any group by Bonferroni-protected planned contrasts. The data for pinprick are presented in the bottom half of Figure 8. The ANOVA revealed that all main and interaction effects were significant, including the three-way interaction ($F(10, 150) = 2.675$, $p = .005$). The significance of this interaction was completely accounted for by other effects in the data that were not related to any specific pre-drug vs. drug contrast in our set of planned comparisons.

Gabapentin. On all days on both sides in the GEL group, gabapentin at 25 mg/kg robustly reduced paw withdrawal responses (Figure 9). The dose of gabapentin used is nearly equivalent to a human dose. Gabapentin significantly reduced the sham pinprick

Figure 8. Celecoxib analgesia results. No analgesic effect of celecoxib was demonstrated; no significant change in paw withdrawals. Graph depicts average of paw withdrawals on pre-drug control days (black) and on paired celecoxib dose days (white). Results of behavior testing show the average of all four light touch allodynia measures (three von Frey fibers and the brush, top graphs) and the mechanical hyperalgesia (pinprick, bottom graphs). Mean and S.E.M. Celecoxib did not significantly reduce paw withdrawal responses on any pair of days for any groups.

Figure 9. Gabapentin analgesia results. Analgesia with reduced paw withdrawals was seen in the GEL group bilaterally. Graph depicts average of paw withdrawals on pre-drug control days (black) and on paired gabapentin dose days (white). Results of behavior testing show the average of all four light touch allodynia measures (three von Frey fibers and the brush, top graphs) and the mechanical hyperalgesia (pinprick, bottom graphs). Mean and S.E.M. *Significant decrease from the paired control day, $p < .05$. Gabapentin also suppressed responding in the sham and control group.

responses on most days. Gabapentin also significantly reduced the level of responding in the control group on days 47 and 76 on both sides. The data for the gabapentin test days using the von Frey fibers and brush are presented in the top half of **Figure 9**. The ANOVA revealed significant main effects and interaction effects except for the three-way interaction. It appears that the group-by-days pattern of responding was similar on the ipsilateral and contralateral sides, therefore, the groups-by-days interaction term is the important one for analysis ($F(10, 150) = 1.929, p = .045$). When the data for the ipsilateral and contralateral sides were combined, we found that gabapentin robustly reduced the GEL group responding on all analgesic test days. For comparison to other figures, asterisks in the top half of **Figure 9** represent significant decreases from the paired pre-drug day by Bonferroni-protected planned contrasts. By this analysis, the comparison for day 114 on the contralateral side for the allodynia measure in the GEL group was not significant. The data for pinprick are presented in the bottom half of **Figure 9**. With the exception of the group-by-side interaction ($p = .055$), all main and interaction effects were significant at $p < .05$ including the three-way interaction ($F(10, 150) = .029, p = .03$).

Duloxetine. Duloxetine at 10 mg/kg reduced pain behaviors bilaterally in the GEL group (Bonferroni-protected contrasts). This dose is markedly less than most prior rat doses⁶⁶, and more than human doses. The bilateral analgesia was similar in the sham group

on D83 and D125, but emerged only after the pain behaviors in them began developing after 2 months. Interestingly, duloxetine did not suppress normal responses to pinprick stimuli in the control group as the gabapentin did. Yet the duloxetine did suppress responses of the contralateral allodynia in the control group. The von Frey fibers and brush data for duloxetine are presented in the top half of **Figure 10**. The ANOVA revealed a significant three-way interaction ($F(10, 150) = 1.99, p = .039$). The data for pinprick are presented in the bottom half of **Figure 10**. The ANOVA revealed that all main and interaction effects were significant except for the three-way interaction. The groups-by-days interaction was significant ($F(10, 150) = 11.358, p < .001$), and the pattern of responding within the groups was similar on the ipsilateral and contralateral sides.

Analgesic responses in sham group

In general, the effects of the different analgesic drugs were similar in the GEL™ and sham groups whenever the sham group demonstrated pain behavior; five of the eight rats in the sham group eventually developed pain behaviors like the GEL group. Morphine demonstrated marked analgesia to the pinprick stimuli on both sides on day 28, but not on later days. This correlates with the waning effect of morphine in the GEL group. Celecoxib did not affect responses in the sham group. As in the GEL group, both gabapentin and duloxetine demonstrated marked analgesia in the sham group.

Figure 10. Duloxetine analgesia results. Analgesia with reduced paw withdrawals was seen in the GEL group bilaterally. A similar response was noted in the last 2 test days of the shams, D83 and D125. Graph depicts average of paw withdrawals on pre-drug control days (black) and on paired duloxetine dose days (white). Results of behavior testing show the average of all four light touch allodynia measures (three von Frey fibers and the brush, top graphs) and the mechanical hyperalgesia (pinprick, bottom graphs). Mean and S.E.M. *Significant decrease from the paired control day, $p < .05$. Duloxetine had less effect than gabapentin on the responses in the control group.

Figure 11. Pain behavior reversed after EPO was injected at the ipsilateral GEL site ($n = 5$). After a perineural infiltration of EPO (200 units in NS) the paw withdrawals to pinprick (hyperalgesia or hypersensitivity) decreased bilaterally to near pre-GEL procedure levels. The groups of "No EPO" control ($n = 5$) and "EPO SC" injected subcutaneously ($n = 4$) continued with robust pain behavior, bilaterally. The + on day 155 signifies two rats in the "EPO at site" group still with pain behavior that had the GEL site on left re-injected with EPO with a different anatomic technique, as described, which caused a decrease in their paw withdrawals to pinprick. $*p < .001$ "EPO at site" vs. "No EPO".

Summary of analgesic effects

Morphine depicted a waning analgesic response over time not related to tolerance, with increasing pain behaviors, suggestive of a developing opioid related hypersensitivity. Celecoxib had no analgesic effect on pain behaviors at any time. By contrast, gabapentin and duloxetine both produced robust analgesia bilaterally in the GEL group during all time periods and in 5/8 shams during the last two drug testing periods. The pain behaviors, when present in the GEL group after D23 and later in the shams after D90, respond to all analgesics similarly.

Reversal of GEL effect with EPO

The pain behavior in the GEL group that had persisted for 4 months was reversed for up to 7 days (end pilot) by the targeted perineural application of epoetin alfa (EPO) in a pilot study, at the end of the investigation (Figure 11). Two of the rats in the "EPO at site" group received a second local EPO injection with a lateral approach on day 155 (seen as a + sign on the left paw results in Figure 11), described in the Materials and Methods section on *Erythropoietin treatment pilot study*. Pinprick behavior data were collected on days 153, 154, 156, 159, and 160. The resulting data were analyzed using a mixed model ANOVA, with the "EPO at site" injection group as the *between subjects* factor (three groups), and *days* as one repeated measures factor (8 days), and *laterality* as a second repeated measures factor (left and right paws). The data are presented in Figure 11. The three-way interaction was not significant, but the groups-by-days interaction was significant ($F(14, 77) = 8.208, p < .001$). As can be seen in the graph, the effect of EPO was nearly identical on the right and left sides and was significant for at least 6 days, end of study. An ANOVA such as this should be interpreted with caution because of several cells with zero variance (there is a ceiling effect of eight paw withdrawals out of eight stimulus presentations of pinprick).

Results of histology

The tissue sections from nine rats randomly selected from each procedure group were blinded, and observed by an independent neuropathologist. These observations were later respectively matched to each of the three groups, with $n = 3$ GEL procedure rats, $n = 3$ sham procedure rats of the 5/8 that displayed late onset robust pain behavior, and $n = 3$ the controls. Details are described in *Histology* within the Materials and Methods section. There were no differences or abnormal findings noted in the tissue sections between the control and the sham procedure animals. The structure of the nerve and surrounding tissue was completely unremarkable (Figure 12).

Within the GEL^{TM} treated group, the histology of the nerves was in stark contrast to the sham and control groups. The gross appearance of the tissue at the time of dissection revealed a discrete area of swelling, or a bulge, along the course of the distal tibial nerve,

Figure 12. Normal tibial nerve histology in both the control and sham procedure groups. Longitudinal sections through the control (a.) and sham (b.) procedure groups reveal normal appearing nerves. The surrounding muscle tissue that was harvested with the nerve also appears to be within normal limits.

in all specimens of the GEL group only. These discrete structures were about twice the diameter of the nerve just distal and proximal to the outcropping. Longitudinal sections through the portion of the tibial nerve that contained these bulges revealed that the swelling is the result of changes within the endoneurium, including evidence of intraneural edema with increased spacing between the neural fascicles, and axonal edema in the fibers within the bulge region (Figure 13 a and b). In addition, there were numerous profiles in which ongoing axonal fragmentation, a hallmark of Wallerian degeneration, was evident (see arrows in Figure 13 a' and c)⁶⁹.

Consistent with ongoing Wallerian degeneration, we observed numerous macrophages within the endoneurium, which phagocytize myelin and axonal debris (Figure 14 a and b large arrows). We also noted a significant leukocyte accumulation in and around the perineurium (Figure 14 b thin arrows).

Findings consistent with any gel residual were not noted in the GEL procedure specimens. In addition to the ongoing Wallerian degeneration observed in the GEL animals, we also noted ongoing axonal regeneration. There were clusters of small, unmyelinated or lightly myelinated fibers growing into the nerves of the GEL cohort only. The grouping of these small fibers is consistent with regeneration, rather than the randomly single-arrayed, unmyelinated fibers that are found in the healthy, homeostatic adult nerve⁷⁰.

Dataset 1. Raw data of NeuroDigm Model of neuropathic pain in mature rat

<http://dx.doi.org/10.5256/f1000research.9544.d137472>

The raw data used for the statistical studies are provided.

Discussion

This nonsurgical NeuroDigm GEL™ aged model appears to mimic many humans complaining of persistent neural pain after soft tissue injuries, by being of gradual onset, persisting for months and lacking deformities or antalgic gait. Two other characteristics have been suggested for relevance in a rodent neuropathic pain model: 1) an analgesic response profile similar to humans and 2) effective analgesia with doses of near human equivalent strengths (14th World Congress IASP 2012; SIG Pain in non-humans species: TW 62 “Closing the Gap between Preclinical and Clinical Studies, Jeffrey Mogil). This socially mature aged outbred rat version of this model of neurogenic pain approaches these analgesic response guidelines.

Mature adult rats^{59,61} were chosen to resemble more closely, human chronic pain patients^{1,71}. The allodynia response in this aged model was weak, while the response to pinprick with hyperalgesia was robust. Younger (270 g) rats created with the NeuroDigm GEL™ method have had robust allodynia over 2 months (Supplementary file S4). Neuropathic pain models in older rats have been recognized as having less pronounced mechanical allodynia than younger ones^{72,73}.

Central sensitivity or neural plasticity is demonstrated in this model after 2–3 weeks by the appearance of elicited pain behavior on the

contralateral side in all GEL procedure rats, as well as the five sham rats that developed the late onset pain behavior. The contralateral spread of pain behaviors in this model raises the possibility that there are central changes as well. Whether this is the result of anatomic changes or the alterations in neural signaling remains to be determined. Central sensitivity with contralateral pain is known to exist in humans with originally unilateral neural pain^{74–77}.

The sham effect with the delayed onset of neuropathic pain was not anticipated, but evaluation of the data depicted a more delayed tissue response (S1) than the GEL group. While the GEL™ was a moderately strong stimulus for tissue repair causing a pain response initially at 23 days (Figure 2), the data shows that the sham fluid was a weaker stimulus for a tissue response, with the gradual consistent onset of pain behavior after 60 days in the five out of eight shams with pain (Figure 2, Figure 6). If this experiment ended prior to 3 months the sham effect would not have been evident. Interestingly, many human neuropathic pain conditions begin months after an injury⁷⁸.

An opioid related hypersensitivity^{79,80} and morphine tolerance is suggested by the data of neural pain behavior developing over time, as seen in the GEL and 5/8 sham animals (Figure 7). Such lack of effective analgesia with morphine over time is characteristic of many neuropathic pain patients^{81–83}. Since each of the three doses of morphine in this study is separated by weeks, the weakening response to morphine does not reflect tolerance but a resistance. This morphine resistance may correlate to the gradual development of nerve injury⁸⁴. Repeated screenings of analgesics over time may determine translational effectiveness⁸⁵. The analgesic responses of this mature GEL model has negative predictive validity for morphine, and positive predictive validity for gabapentin and duloxetine.

Erythropoietin^{86–89}, methylprednisolone⁹⁰, glucocorticoids in general⁹¹, and ARA290, an erythropoietin derived tissue repair peptide^{92–94}, as well as other biologics⁹⁵, are known to have neuroprotective effects systemically and locally in nerve injury rodent models. The local erythropoietin dose at the neural lesion in this model may integrate the tissue repair ability of erythropoietin⁹⁶ in alleviating pain behavior. In this model the EPO injection acts similar to a diagnostic and therapeutic peripheral nerve block.

The targeted local injection of an erythropoietin analog in this study appears to support the localized “ectopic” and “generator” theories for the persistence of neural pain. The unilateral application of erythropoietin appears to have markedly reduced, for at least 6 days (end of study), the bilateral pain behavior of mechanical hyperalgesia (pinprick). The focal neural swelling seen on the distal tibial nerve in the GEL group acts like a mid-axon nociceptive stimulus, maintaining the pain behavior until the localized erythropoietin healed the neural lesion.

An unanticipated feature suggested in our 5-month study is the evidence for habituation to the von Frey and brush stimuli. In this study, the light touch stimuli (von Frey, brush) have less painful significance than the pinprick, so their responses may be susceptible to habituation (Figure 3, Figure 4). We have not been able to

Figure 13. Edema and Wallerian degeneration in the nerves of GEL procedure rats. Panels **a.** and **b.** reflect the gross swelling seen in the tibial nerves upon dissection. The brackets in panels **a.** and **b.** denote the prominent areas of swelling in the GEL nerves. The axons within the swollen area are themselves swollen. The diameters of the axons in this distended area of the nerve are approximately twice the diameter of the contiguous axons proximal or distal (not shown) to it. The arrowheads point to axonal debris in panel **a'**, which is a magnification of the area within the box in panel **a.** Macrophage-engulfed myelin and axonal debris (panel **c.**, arrows) is further evidence of ongoing Wallerian degeneration.

Figure 14. Simultaneous Wallerian degeneration and axonal regeneration is evident in the GEL group nerves. Intraneural (large arrows **a.** and **b.**) and perineural leukocytic infiltration (thin arrows, **b.**) is seen in the GEL group nerves. Notably, there are numerous profiles of regeneration axons in the same nerve (panel **c.** and **c'**) at the same proximal/distal level, suggesting ongoing nerve remodeling in the GEL procedure animals. Panel **c'** is a higher power magnification of the clusters of small, unmyelinated axons within the box in panel **c.**

locate any specific study of habituation to von Frey or brush stimuli. Ipsilateral responses to light touch stimuli in the GEL group appeared to diminish in post procedure periods 4 and 5. By contrast, the sham group tended to remain the same or increase during the same time periods, as some of them developed pain behavior. We did not observe any evidence of habituation to the pinprick stimulus in any group.

The nerve specimens used in this study were processed at end of the near 6-month study, when chronic tissue changes dominated, without evidence of acute inflammation⁹⁷. The histological changes seen were restricted to the NeuroDigm GEL™ procedure group and are consistent with ongoing tissue remodeling in the area where the GEL was placed. Specifically, there was evidence of both Wallerian degeneration and axonal regeneration, hallmarks of nerve remodeling. The GEL evoked remodeling of extraneural matrix tissue may result in nerve compression⁹⁸, resulting in neural remodeling with the delayed onset of pain⁴². Also consistent with ongoing nerve remodeling is the observed inflammation with leukocytic infiltration, seen in both the endoneurial environment and the extraneural space.

Notably, a large number of tightly packed unmyelinated fibers were within the nerves of the GEL procedure animals, consistent with regeneration. The increased number of these fibers and their unusual clustered appearance raises the issues of adequate insulation and the possibility that some of the pain behavior might be due to ephaptic transmission^{99–101}. Neural regeneration with ephaptic transmission is likely the underlying cause of the both the Tinel's sign¹⁰² observed in some patients at sites of neural compression due to entrapment, and also the Pinch Reflex Test found at sites of regenerating peripheral nerves in experimental rodents^{103,104}.

The histology demonstrates that the light microscopy anatomical response to the GEL procedure is restricted to a focal area of neural and axonal edema with neuroinflammation in the tibial nerve, yet the behavioral effects are widespread. Light microscopy showed that the three shams (from the 5/8 with robust pain behavior) had no visible anatomic changes on histology, similar to the paclitaxel model¹⁰⁵. Additional studies are needed in which the effects of the GEL™ induced distal mononeuritis are explored on the brain, spinal cord and the dorsal root ganglion.

The mechanisms that lead to the anatomic and behavioral changes are not yet determined. However, it is likely that the neural remodeling⁹¹ seen in our model's neuritis reflects many of the ongoing changes that are seen with chronic pain following peripheral trauma¹⁰⁶. The findings in our study are consistent with prior findings found in neural biopsies of humans with severe persistent pain due to known nerve entrapments^{102,107,108}. The creation of this model allows for further in-depth studies to understand the events around the establishment of chronic neural pain following minimal trauma as seen in humans.

Conclusion

Our model simulates the gradual sequelae of a soft tissue injury with a focal matrix compression (a pinch) causing an ectopic neural site with persistent neurogenic pain^{109,110}. Gradual perineural changes of the extracellular matrix and scarring¹¹¹ can cause such focal compressions on a nerve⁴². Recently, a lesion of the somatosensory system, or a disease, has been needed for the definition of neuropathic pain¹¹². This model provides an extended temporal window into an occult focal neural lesion as a naturally occurring soft tissue disease¹¹³.

The GEL method supports the 3Rs initiative for refinement in the humane use of animals, and if predictive at determining analgesia may lead to a reduction in animals used. The tissue reaction used in this refinement is an encoded response occurring after any tissue injury in all vertebrates^{114,115}. The last stage of this repair process is tissue remodeling (S1)^{113,116} which may be a unifying etiology¹¹⁷ to target in many complex neuropathic pain syndromes.

We have used tissue repair in this model to create neuropathic pain, and also to treat it. Performing analgesic studies on a mature adult rat with a tissue matrix neural lesion may help reveal the analgesic potential of an agent for humans with neuropathic pain. The refined¹¹⁸ NeuroDigm GEL™ Model has an accessible neural biomimetic target for translational studies exploring cell signaling, biomarkers, analgesics, detection devices, biologic treatments and alternatives to opiates.

Data availability

Dataset

F1000Research: Dataset 1. Raw data of NeuroDigm Model of neuropathic pain in mature rat, [10.5256/f1000research.9544.d137472](https://doi.org/10.5256/f1000research.9544.d137472)¹¹⁹

Author contributions

Conceived and designed the experiments: MRH DAF DEW JLB. Performed the experiments: MRH RMD DEW. Analyzed the data: DAF DEW MRH. Wrote the paper: MRH DAF RMD DEW JLB. All authors agreed to the final content of the article.

Competing interests

MRH is an officer and stockholder in NeuroDigm Corporation.

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References

1. Percie du Sert N, Rice AS: **Improving the translation of analgesic drugs to the clinic: animal models of neuropathic pain.** *Br J Pharmacol.* 2014; **171**(12): 2951–63.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
2. de Mos M, de Bruijn AG, Huygen FJ, *et al.*: **The incidence of complex regional pain syndrome: a population-based study.** *Pain.* 2007; **129**(1-2): 12–20.
[PubMed Abstract](#) | [Publisher Full Text](#)
3. Dieleman JP, Kerklaan J, Huygen FJ, *et al.*: **Incidence rates and treatment of neuropathic pain conditions in the general population.** *Pain.* 2008; **137**(3): 681–8.
[PubMed Abstract](#) | [Publisher Full Text](#)
4. Sandroni P, Benrud-Larson LM, McClelland RL, *et al.*: **Complex regional pain syndrome type I: incidence and prevalence in Olmsted county, a population-based study.** *Pain.* 2003; **103**(1–2): 199–207.
[PubMed Abstract](#) | [Publisher Full Text](#)
5. Payne R: **Neuropathic Pain Syndromes, with special reference to Causalgia and Reflex Sympathetic Dystrophy.** *The Clinical Journal of Pain.* 1986; **2**(1): 59–73.
[Reference Source](#)
6. Elkind AH: **Headache and facial pain associated with head injury.** *Otolaryngol Clin North Am.* 1989; **22**(6): 1251–71.
[PubMed Abstract](#)
7. Freund B, Schwartz M: **Post-traumatic myofascial pain of the head and neck.** *Curr Pain Headache Rep.* 2002; **6**(5): 361–9.
[PubMed Abstract](#) | [Publisher Full Text](#)
8. Rho RH, Brewer RP, Lamer TJ, *et al.*: **Complex regional pain syndrome.** *Mayo Clin Proc.* 2002; **77**(2): 174–80.
[PubMed Abstract](#) | [Publisher Full Text](#)
9. Roberts WJ: **A hypothesis on the physiological basis for causalgia and related pains.** *Pain.* 1986; **24**(3): 297–311.
[PubMed Abstract](#) | [Publisher Full Text](#)
10. Rotter R, Kuhn C, Stratos I, *et al.*: **Erythropoietin enhances the regeneration of traumatized tissue after combined muscle-nerve injury.** *J Trauma Acute Care Surg.* 2012; **72**(6): 1567–75.
[PubMed Abstract](#) | [Publisher Full Text](#)
11. Schwartzman RJ, Grothusen J, Kiefer TR, *et al.*: **Neuropathic central pain: epidemiology, etiology, and treatment options.** *Arch Neurol.* 2001; **58**(10): 1547–50.
[PubMed Abstract](#) | [Publisher Full Text](#)
12. Schwartzman RJ, Maleki J: **Postinjury neuropathic pain syndromes.** *Med Clin North Am.* 1999; **83**(3): 597–626.
[PubMed Abstract](#) | [Publisher Full Text](#)
13. Seale KS: **Reflex sympathetic dystrophy of the lower extremity.** *Clin Orthop Relat Res.* 1989; **243**: 80–5.
[PubMed Abstract](#)
14. van der Laan L, Goris RJ: **Reflex sympathetic dystrophy. An exaggerated regional inflammatory response?** *Hand Clin.* 1997; **13**(3): 373–85.
[PubMed Abstract](#)
15. Wasner G, Schattschneider J, Binder A, *et al.*: **Complex regional pain syndrome—diagnostic, mechanisms, CNS involvement and therapy.** *Spinal Cord.* 2003; **41**(2): 61–75.
[PubMed Abstract](#) | [Publisher Full Text](#)
16. Wilson PR: **Post-traumatic upper extremity reflex sympathetic dystrophy. Clinical course, staging, and classification of clinical forms.** *Hand Clin.* 1997; **13**(3): 367–72.
[PubMed Abstract](#)
17. Hooshmand H: **Chronic pain: reflex sympathetic dystrophy, prevention, and management.** Boca Raton, Florida: CRC Press; 1993; 201.
[Reference Source](#)
18. Oaklander AL: **Role of minimal distal nerve injury in complex regional pain syndrome-I.** *Pain Med.* 2010; **11**(8): 1251–6.
[PubMed Abstract](#) | [Publisher Full Text](#)
19. Ali Z, Ringkamp M, Hartke TV, *et al.*: **Uninjured C-fiber nociceptors develop spontaneous activity and alpha-adrenergic sensitivity following L6 spinal nerve ligation in monkey.** *J Neurophysiol.* 1999; **81**(2): 455–66.
[PubMed Abstract](#)
20. Campbell JN, Meyer RA: **Mechanisms of neuropathic pain.** *Neuron.* 2006; **52**(1): 77–92.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
21. Devor M: **Nerve pathophysiology and mechanisms of pain in causalgia.** *J Auton Nerv Syst.* 1983; **7**(3–4): 371–84.
[PubMed Abstract](#) | [Publisher Full Text](#)
22. Devor M: **Neuropathic pain and injured nerve: peripheral mechanisms.** *Br Med Bull.* 1991; **47**(3): 619–30.
[PubMed Abstract](#)
23. Finnerup NB, Baastrop C: **Spinal cord injury pain: mechanisms and management.** *Curr Pain Headache Rep.* 2012; **16**(3): 207–16.
[PubMed Abstract](#) | [Publisher Full Text](#)
24. Finnerup NB, Jensen TS: **Spinal cord injury pain—mechanisms and treatment.** *Eur J Neurol.* 2004; **11**(2): 73–82.
[PubMed Abstract](#) | [Publisher Full Text](#)
25. Han HC, Lee DH, Chung JM: **Characteristics of ectopic discharges in a rat neuropathic pain model.** *Pain.* 2000; **84**(2–3): 253–61.
[PubMed Abstract](#) | [Publisher Full Text](#)
26. Hu P, McLachlan EM: **Macrophage and lymphocyte invasion of dorsal root ganglia after peripheral nerve lesions in the rat.** *Neuroscience.* 2002; **112**(1): 23–38.
[PubMed Abstract](#) | [Publisher Full Text](#)
27. Jensen TS, Baron R: **Translation of symptoms and signs into mechanisms in neuropathic pain.** *Pain.* 2003; **102**(1–2): 1–8.
[PubMed Abstract](#) | [Publisher Full Text](#)
28. Woolf CJ: **The pathophysiology of peripheral neuropathic pain—abnormal peripheral input and abnormal central processing.** *Acta Neurochir Suppl (Wien).* 1993; **58**: 125–30.
[PubMed Abstract](#) | [Publisher Full Text](#)
29. Costigan M, Scholz J, Woolf CJ: **Neuropathic pain: a maladaptive response of the nervous system to damage.** *Annu Rev Neurosci.* 2009; **32**: 1–32.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
30. DeLeo JA, Yezierski RP: **The role of neuroinflammation and neuroimmune activation in persistent pain.** *Pain.* 2001; **90**(1–2): 1–6.
[PubMed Abstract](#) | [Publisher Full Text](#)
31. Bouhassira D, Attal N: **Diagnosis and assessment of neuropathic pain: the saga of clinical tools.** *Pain.* 2011; **152**(3 Suppl): S74–83.
[PubMed Abstract](#) | [Publisher Full Text](#)
32. Oaklander AL, Wilson PR, Moskovitz PA, *et al.*: **Response to “A new definition of neuropathic pain”.** *Pain.* 2012; **153**(4): 934–5; author reply 935–6.
[PubMed Abstract](#) | [Publisher Full Text](#)
33. Dille A, Greening J, Walker-Bone K, *et al.*: **Magnetic resonance imaging signal hyperintensity of neural tissues in diffuse chronic pain syndromes: a pilot study.** *Muscle Nerve.* 2011; **44**(6): 981–4.
[PubMed Abstract](#) | [Publisher Full Text](#)
34. Filler A: **Magnetic resonance neurography and diffusion tensor imaging: origins, history, and clinical impact of the first 50,000 cases with an assessment of efficacy and utility in a prospective 5000-patient study group.** *Neurosurgery.* 2009; **65**(4 suppl): A29–43.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
35. Filler AG, Bell BA: **Axonal transport, imaging, and the diagnosis of nerve compression.** *Br J Neurosurg.* 1992; **6**(4): 293–5.
[PubMed Abstract](#) | [Publisher Full Text](#)
36. Lawande AD, Warriar SS, Joshi MS: **Role of ultrasound in evaluation of peripheral nerves.** *Indian J Radiol Imaging.* 2014; **24**(3): 254–8.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
37. Simon NG, Narvid J, Cage T, *et al.*: **Visualizing axon regeneration after peripheral nerve injury with magnetic resonance tractography.** *Neurology.* 2014; **83**(15): 1382–4.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
38. Tung KW, Behera D, Biswal S: **Neuropathic pain mechanisms and imaging.** *Semin Musculoskelet Radiol.* 2015; **19**(2): 103–11.
[PubMed Abstract](#) | [Publisher Full Text](#)
39. Vaeggemose M, Ringgaard S, Ejskjaer N, *et al.*: **Magnetic resonance imaging may be used for early evaluation of diabetic peripheral polyneuropathy.** *J Diabetes Sci Technol.* 2015; **9**(1): 162–3.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
40. Jacobson JA, Wilson TJ, Yang LJ: **Sonography of Common Peripheral Nerve Disorders With Clinical Correlation.** *J Ultrasound Med.* 2016; **35**(4): 683–93.
[PubMed Abstract](#) | [Publisher Full Text](#)
41. Kerasnoudis A: **Ultrasound visualization of nerve remodeling after strenuous exercise.** *Muscle Nerve.* 2016; **53**(2): 320–4.
[PubMed Abstract](#) | [Publisher Full Text](#)
42. Myers RR: **1994 ASRA Lecture. The pathogenesis of neuropathic pain.** *Reg Anesth.* 1995; **20**(3): 173–84.
[PubMed Abstract](#)
43. Bowsher D: **Trigeminal neuralgia: an anatomically oriented review.** *Clin Anat.* 1997; **10**(6): 409–15.
[PubMed Abstract](#) | [Publisher Full Text](#)
44. Hinz B, Phan SH, Thannickal VJ, *et al.*: **Recent developments in myofibroblast biology: paradigms for connective tissue remodeling.** *Am J Pathol.* 2012; **180**(4): 1340–55.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
45. Velnar T, Bailey T, Smrkolj V: **The wound healing process: an overview of the cellular and molecular mechanisms.** *J Int Med Res.* 2009; **37**(5): 1528–42.
[PubMed Abstract](#) | [Publisher Full Text](#)
46. Zochodne DW: **The microenvironment of injured and regenerating peripheral nerves.** *Muscle Nerve Suppl.* 2000; **9**(Supplement S9): S33–8.
[PubMed Abstract](#) | [Publisher Full Text](#)
47. Ogawa R: **Mechanobiology of scarring.** *Wound Repair Regen.* 2011; **19**(Suppl 1): s2–9.
[PubMed Abstract](#) | [Publisher Full Text](#)
48. Scholz J, Woolf CJ: **The neuropathic pain triad: neurons, immune cells and glia.** *Nat Neurosci.* 2007; **10**(11): 1361–8.
[PubMed Abstract](#) | [Publisher Full Text](#)

49. Mogil JS: **Animal models of pain: progress and challenges.** *Nat Rev Neurosci.* 2009; **10**(4): 283–94.
[PubMed Abstract](#) | [Publisher Full Text](#)
50. Mogil JS, Davis KD, Derbyshire SW: **The necessity of animal models in pain research.** *Pain.* 2010; **151**(1): 12–7.
[PubMed Abstract](#) | [Publisher Full Text](#)
51. Ansell DM, Holden KA, Hardman MJ: **Animal models of wound repair: Are they cutting it?** *Exp Dermatol.* 2012; **21**(8): 581–5.
[PubMed Abstract](#) | [Publisher Full Text](#)
52. Peplow PV, Chung TY, Baxter GD: **Photodynamic modulation of wound healing: a review of human and animal studies.** *Photomed Laser Surg.* 2012; **30**(3): 118–48.
[PubMed Abstract](#) | [Publisher Full Text](#)
53. van der Worp HB, Howells DW, Sena ES, *et al.*: **Can animal models of disease reliably inform human studies?** *PLoS Med.* 2010; **7**(3): e1000245.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
54. Jaggi AS, Jain V, Singh N: **Animal models of neuropathic pain.** *Fundam Clin Pharmacol.* 2011; **25**(1): 1–28.
[PubMed Abstract](#) | [Publisher Full Text](#)
55. Abdo H: **Gait Analysis and Therapeutic Application of Carbon Monoxide in a Rodent Model of Complex Regional Pain Syndrome Type-1.** *Electronic Thesis and Dissertation Repository: The University of Western Ontario.* 2015.
[Reference Source](#)
56. Kim SH, Chung JM: **An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat.** *Pain.* 1992; **50**(3): 355–63.
[PubMed Abstract](#) | [Publisher Full Text](#)
57. Bennett GJ, Xie YK: **A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man.** *Pain.* 1988; **33**(1): 87–107.
[PubMed Abstract](#) | [Publisher Full Text](#)
58. Decosterd I, Woolf CJ: **Spared nerve injury: an animal model of persistent peripheral neuropathic pain.** *Pain.* 2000; **87**(2): 149–58.
[PubMed Abstract](#) | [Publisher Full Text](#)
59. Sengupta P: **The Laboratory Rat: Relating Its Age With Human's.** *Int J Prev Med.* 2013; **4**(6): 624–30.
[PubMed Abstract](#) | [Free Full Text](#)
60. Andreollo NA, Santos EF, Araújo MR, *et al.*: **Rat's age versus human's age: what is the relationship?** *Arq Bras Cir Dig.* 2012; **25**(1): 49–51.
[PubMed Abstract](#) | [Publisher Full Text](#)
61. Quinn R: **Comparing rat's to human's age: how old is my rat in people years?** *Nutrition.* United States, 2005; **21**(6): 775–7.
[PubMed Abstract](#) | [Publisher Full Text](#)
62. Fitts DA: **Improved stopping rules for the design of efficient small-sample experiments in biomedical and biobehavioral research.** *Behav Res Methods.* 2010; **42**(1): 3–22.
[PubMed Abstract](#) | [Publisher Full Text](#)
63. Fitts DA: **Ethics and animal numbers: informal analyses, uncertain sample sizes, inefficient replications, and type I errors.** *J Am Assoc Lab Anim Sci.* 2011; **50**(4): 445–53.
[PubMed Abstract](#) | [Free Full Text](#)
64. Achneck HE, Sileshi B, Jamiolkowski RM, *et al.*: **A comprehensive review of topical hemostatic agents: efficacy and recommendations for use.** *Ann Surg.* 2010; **251**(2): 217–28.
[PubMed Abstract](#) | [Publisher Full Text](#)
65. Drury JL, Mooney DJ: **Hydrogels for tissue engineering: scaffold design variables and applications.** *Biomaterials.* 2003; **24**(24): 4337–51.
[PubMed Abstract](#) | [Publisher Full Text](#)
66. Munro G, Storm A, Hansen MK, *et al.*: **The combined predictive capacity of rat models of allergen-induced and neuropathic hypersensitivity to clinically used analgesics varies with nociceptive endpoint and consideration of locomotor function.** *Pharmacol Biochem Behav.* 2012; **101**(3): 465–78.
[PubMed Abstract](#) | [Publisher Full Text](#)
67. Brabb T, Carbone L, Snyder J, *et al.*: **Institutional animal care and use committee considerations for animal models of peripheral neuropathy.** *ILAR J.* 2014; **54**(3): 329–37.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
68. Sorge RE, Martin LJ, Isbester KA, *et al.*: **Olfactory exposure to males, including men, causes stress and related analgesia in rodents.** *Nat Methods.* 2014; **11**(6): 629–32.
[PubMed Abstract](#) | [Publisher Full Text](#)
69. Griffin JW, George EB, Chaudhry V: **Wallerian degeneration in peripheral nerve disease.** *Baillieres Clin Neurol.* 1996; **5**(1): 65–75.
[PubMed Abstract](#)
70. Gondré M, Burrola P, Weinstein DE: **Accelerated nerve regeneration mediated by Schwann cells expressing a mutant form of the POU protein SCIP.** *J Cell Biol.* 1998; **141**(2): 493–501.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
71. Pickering G, Jourdan D, Millescamp M, *et al.*: **Age-related impact of neuropathic pain on animal behaviour.** *Eur J Pain.* 2006; **10**(8): 749–55.
[PubMed Abstract](#) | [Publisher Full Text](#)
72. Chung JM, Choi Y, Yoon YW, *et al.*: **Effects of age on behavioral signs of neuropathic pain in an experimental rat model.** *Neurosci Lett.* 1995; **183**(1–2): 54–7.
[PubMed Abstract](#) | [Publisher Full Text](#)
73. Crisp T, Giles JR, Cruce WL, *et al.*: **The effects of aging on thermal hyperalgesia and tactile-evoked allodynia using two models of peripheral mononeuropathy in the rat.** *Neurosci Lett.* 2003; **339**(2): 103–6.
[PubMed Abstract](#) | [Publisher Full Text](#)
74. Shenker N, Haigh R, Roberts E, *et al.*: **A review of contralateral responses to a unilateral inflammatory lesion.** *Rheumatology (Oxford).* 2003; **42**(11): 1279–86.
[PubMed Abstract](#) | [Publisher Full Text](#)
75. Shenker NG, Haigh RC, Mapp PI, *et al.*: **Contralateral hyperalgesia and allodynia following intradermal capsaicin injection in man.** *Rheumatology (Oxford).* 2008; **47**(9): 1417–21.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
76. Schmid AB, Nee RJ, Coppieters MW: **Reappraising entrapment neuropathies—mechanisms, diagnosis and management.** *Man Ther.* 2013; **18**(6): 449–57.
[PubMed Abstract](#) | [Publisher Full Text](#)
77. Maleki J, LeBel AA, Bennett GJ, *et al.*: **Patterns of spread in complex regional pain syndrome, type I (reflex sympathetic dystrophy).** *Pain.* 2000; **88**(3): 259–66.
[PubMed Abstract](#) | [Publisher Full Text](#)
78. Schott GD: **Delayed onset and resolution of pain: some observations and implications.** *Brain.* 2001; **124**(Pt 6): 1067–76.
[PubMed Abstract](#) | [Publisher Full Text](#)
79. Attal N, Chen YL, Kayser V, *et al.*: **Behavioural evidence that systemic morphine may modulate a phasic pain-related behaviour in a rat model of peripheral mononeuropathy.** *Pain.* 1991; **47**(1): 65–70.
[PubMed Abstract](#) | [Publisher Full Text](#)
80. Ossipov MH, Lopez Y, Nichols ML, *et al.*: **The loss of antinociceptive efficacy of spinal morphine in rats with nerve ligation injury is prevented by reducing spinal afferent drive.** *Neurosci Lett.* 1995; **199**(2): 87–90.
[PubMed Abstract](#) | [Publisher Full Text](#)
81. Freynhagen R, Geisslinger G, Schug SA: **Opioids for chronic non-cancer pain.** *BMJ.* 2013; **346**: f2937.
[PubMed Abstract](#) | [Publisher Full Text](#)
82. Fields HL: **The doctor's dilemma: opiate analgesics and chronic pain.** *Neuron.* 2011; **69**(4): 591–4.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
83. Horvath RJ, Romero-Sandoval EA, De Leo JA: **Glial Modulation in Pain States: Translation into humans.** In Kruger L, Light AR, editors. *Translational Pain Research: From Mouse to Man.* Boca Raton, FL: CRC Press, 2010; 215–234.
[PubMed Abstract](#)
84. Raghavendra V, Rutkowski MD, De Leo JA: **The role of spinal neuroimmune activation in morphine tolerance/hyperalgesia in neuropathic and sham-operated rats.** *J Neurosci.* 2002; **22**(22): 9980–9.
[PubMed Abstract](#)
85. Hama A, Sagen J: **Altered antinociceptive efficacy of tramadol over time in rats with painful peripheral neuropathy.** *Eur J Pharmacol.* 2007; **559**(1): 32–7.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
86. Campana WM, Li X, Shubayev VI, *et al.*: **Erythropoietin reduces Schwann cell TNF-alpha, Wallerian degeneration and pain-related behaviors after peripheral nerve injury.** *Eur J Neurosci.* 2006; **23**(3): 617–26.
[PubMed Abstract](#) | [Publisher Full Text](#)
87. Elfar JC, Jacobson JA, Puzas JE, *et al.*: **Erythropoietin accelerates functional recovery after peripheral nerve injury.** *J Bone Joint Surg Am.* 2008; **90**(8): 1644–53.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
88. Li X, Gonias SL, Campana WM: **Schwann cells express erythropoietin receptor and represent a major target for Epo in peripheral nerve injury.** *Glia.* 2005; **51**(4): 254–65.
[PubMed Abstract](#) | [Publisher Full Text](#)
89. Campana WM, Myers RR: **Exogenous erythropoietin protects against dorsal root ganglion apoptosis and pain following peripheral nerve injury.** *Eur J Neurosci.* 2003; **18**(6): 1497–506.
[PubMed Abstract](#) | [Publisher Full Text](#)
90. Johansson A, Bennett GJ: **Effect of local methylprednisolone on pain in a nerve injury model. A pilot study.** *Reg Anesth.* 1997; **22**(1): 59–65.
[PubMed Abstract](#) | [Publisher Full Text](#)
91. Devor M: **The pathophysiology of damaged peripheral nerves.** In: Wall PD, Melzak R, editors. *Textbook of Pain.* 3rd ed. London: Churchill-Livingston; 1994; 79–100.
92. Dilley A: **ARA290 in a rat model of inflammatory pain.** *Methods Mol Biol.* 2013; **982**: 213–25.
[PubMed Abstract](#) | [Publisher Full Text](#)
93. Pulman KG, Smith M, Mengozzi M, *et al.*: **The erythropoietin-derived peptide ARA290 reverses mechanical allodynia in the neuritis model.** *Neuroscience.* 2013; **233**: 174–83.
[PubMed Abstract](#) | [Publisher Full Text](#)
94. Swartjes M, Niesters M, Dahan A: **Assessment of allodynia relief by tissue-protective molecules in a rat model of nerve injury-induced neuropathic pain.** *Methods Mol Biol.* 2013; **982**: 187–95.
[PubMed Abstract](#) | [Publisher Full Text](#)
95. Richards N, McMahon SB: **Targeting novel peripheral mediators for the treatment of chronic pain.** *Br J Anaesth.* 2013; **111**(1): 46–51.
[PubMed Abstract](#) | [Publisher Full Text](#)
96. Brines M: **Discovery of a master regulator of injury and healing: tipping the outcome from damage toward repair.** *Mol Med.* 2014; **20**(Suppl 1): S10–6.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)

97. Ji RR, Xu ZZ, Gao YJ: **Emerging targets in neuroinflammation-driven chronic pain.** *Nat Rev Drug Discov.* 2014; **13**(7): 533–48.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
98. Hayashi A, Pannucci C, Moradzadeh A, *et al.*: **Axotomy or compression is required for axonal sprouting following end-to-side neurorrhaphy.** *Exp Neurol.* 2008; **211**(2): 539–50.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
99. Krafft RM: **Trigeminal neuralgia.** *Am Fam Physician.* 2008; **77**(9): 1291–6.
[PubMed Abstract](#)
100. Ueda H: **Peripheral mechanisms of neuropathic pain - involvement of lysophosphatidic acid receptor-mediated demyelination.** *Mol Pain.* 2008; **4**: 11.
[PubMed Abstract](#) | [Free Full Text](#)
101. Seltzer Z, Devor M: **Ephaptic transmission in chronically damaged peripheral nerves.** *Neurology.* 1979; **29**(7): 1061–4.
[PubMed Abstract](#) | [Publisher Full Text](#)
102. Mackinnon SE, Dellon AL, Hudson AR, *et al.*: **Chronic human nerve compression—a histological assessment.** *Neuropathol Appl Neurobiol.* 1986; **12**(6): 547–65.
[PubMed Abstract](#) | [Publisher Full Text](#)
103. Bisby MA, Pollock B: **Increased regeneration rate in peripheral nerve axons following double lesions: enhancement of the conditioning lesion phenomenon.** *J Neurobiol.* 1983; **14**(6): 467–72.
[PubMed Abstract](#) | [Publisher Full Text](#)
104. Danielsson P, Dahlin L, Povlsen B: **Tubulization increases axonal outgrowth of rat sciatic nerve after crush injury.** *Exp Neurol.* 1996; **139**(2): 238–43.
[PubMed Abstract](#) | [Publisher Full Text](#)
105. Polomano RC, Mannes AJ, Clark US, *et al.*: **A painful peripheral neuropathy in the rat produced by the chemotherapeutic drug, paclitaxel.** *Pain.* 2001; **94**(3): 293–304.
[PubMed Abstract](#) | [Publisher Full Text](#)
106. Martín YB, Herradón G, Ezquerro L: **Uncovering new pharmacological targets to treat neuropathic pain by understanding how the organism reacts to nerve injury.** *Curr Pharm Des.* 2011; **17**(5): 434–48.
[PubMed Abstract](#) | [Publisher Full Text](#)
107. Mackinnon SE, Dellon AL, Hudson AR, *et al.*: **Histopathology of compression of the superficial radial nerve in the forearm.** *J Hand Surg Am.* 1986; **11**(2): 206–10.
[PubMed Abstract](#) | [Publisher Full Text](#)
108. Rempel D, Dahlin L, Lundborg G: **Pathophysiology of nerve compression syndromes: response of peripheral nerves to loading.** *J Bone Joint Surg Am.* 1999; **81**(11): 1600–10.
[PubMed Abstract](#)
109. Sommer C, Galbraith JA, Heckman HM, *et al.*: **Pathology of experimental compression neuropathy producing hyperesthesia.** *J Neuropathol Exp Neurol.* 1993; **52**(3): 223–33.
[PubMed Abstract](#) | [Publisher Full Text](#)
110. Schmid AB, Coppieters MW, Ruitenberg MJ, *et al.*: **Local and remote immune-mediated inflammation after mild peripheral nerve compression in rats.** *J Neuropathol Exp Neurol.* 2013; **72**(7): 662–80.
[PubMed Abstract](#) | [Publisher Full Text](#)
111. Cox TR, Erler JT: **Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer.** *Dis Model Mech.* 2011; **4**(2): 165–78.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
112. Jensen TS, Baron R, Haanpää M, *et al.*: **A new definition of neuropathic pain.** *Pain.* Netherlands, 2011; **152**(10): 2204–5.
[PubMed Abstract](#) | [Publisher Full Text](#)
113. Handorf AM, Zhou Y, Halanski MA, *et al.*: **Tissue stiffness dictates development, homeostasis, and disease progression.** *Organogenesis.* 2015; **11**(1): 1–15.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
114. Coleman LS: **A stress repair mechanism that maintains vertebrate structure during stress.** *Cardiovasc Hematol Disord Drug Targets.* 2010; **10**(2): 111–37.
[PubMed Abstract](#) | [Publisher Full Text](#)
115. Eming SA, Hammerschmidt M, Krieg T, *et al.*: **Interrelation of immunity and tissue repair or regeneration.** *Semin Cell Dev Biol.* 2009; **20**(5): 517–27.
[PubMed Abstract](#) | [Publisher Full Text](#)
116. Mason BN, Califano JP, Reinhart-King CA: **Matrix Stiffness: A Regulator of Cellular Behavior and Tissue Formation.** S.K. Bhatia, editor: Springer Science; 2012; 19–37.
[Publisher Full Text](#)
117. Hama AT, Borsook D: **Behavioral and pharmacological characterization of a distal peripheral nerve injury in the rat.** *Pharmacol Biochem Behav.* 2005; **81**(1): 170–81.
[PubMed Abstract](#) | [Publisher Full Text](#)
118. Flecknell P: **Replacement, reduction and refinement.** *ALTEX.* 2002; **19**(2): 73–8.
[PubMed Abstract](#)
119. Hannaman MR, Fitts DA, Doss RM, *et al.*: **Dataset 1 in: The refined biomimetic NeuroDigm GEL™ Model of neuropathic pain in the mature rat.** *F1000Research.* 2016.
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The research article "The refined biomimetic NeuroDigm GEL™ Model of neuropathic pain in the mature rat" describes a new neuropathic pain animal model designed to be more relevant to the development of chronic neuropathic pain in humans. The authors describe a model using mature adult rats with a percutaneous implant of GEL™ into the tibial nerve. The model is characterised for 5 months by assessing pain-related behavioural responses to mechanical stimuli and the effect of morphine, celecoxib, gabapentin and duloxetine. Histology of the nerve was also assessed. A pilot study is also reported assessing the pain-related behavioural response to the injection of human erythropoietin.

The rationale for the development of a model of neuropathic pain that more closely mimics the human condition is sound. I agree that the most commonly used neuropathic pain models do not mirror the pathophysiology of the delayed onset of neural pain without debility as seen in many neuropathic pain patients. The authors provide detailed description of their methods and rationale and include an ARRIVE guidelines checklist.

I am concerned that the Sham animals are not used as controls to characterise this model. The authors acknowledge that the sham animals are not homogenous in their pain-related behavioural responses (5 out of 8 animals develop pinprick-induced pain-related behaviours). However, I believe that this is the most appropriate control for this new model of neuropathic pain.

I disagree with the claim that this model meets the NC3Rs criteria for refinement. Refinement refers to methods that minimise the pain, suffering, distress or lasting harm that may be experienced by animals. The longer duration of this model and the development of similar pain-related behaviours as observed in other neuropathic pain models does not meet refinement criteria.

Specific recommendations:

Title

Description of the model as refined needs to be clarified in the article. How is this a refined model?

Abstract

Research objectives should be stated in the abstract.

Strain of animal should be stated in the abstract.

I do not think the results of the pilot study should be stated in the conclusions of the abstract. The main study characterising the model, which is appropriately powered, should be the focus.

Introduction and background

It would be useful to use more specific references for the type of pain that this model is meant to be modelling.

Materials and Methods

Animals were randomly assigned to groups as stated in the Methods section Study Design Paragraph. Please state how animals were randomly allocated to group.
Also, state whether animals were randomly assigned to analgesic treatment groups and how this was carried out.

Results

The data supports the claim that the model does develop pain-related behaviours that develop gradually and persist for months compared to control animals. However, should the comparison be to sham animals?

Do the analgesic responses reflect human responses?

All figures: For presentation of results in the figures I recommend the used of standard deviations not standard error of the mean¹.

Figure 5: The Sham animals also show an increase in pain behaviour from baseline and this should be indicated with asterisks.

Figure 7-10: How many animals were tested in the analgesic drug experiments? This should be clearly stated in the results section and in the figure legend. Were the same animals used for each drug? If so, this should be clearly stated.

The authors give a thorough and transparent description of their data and analysis choices. However, I recommend that a statistician assesses the statistical methods. For example, I question the use of the Fisher's Protected Least Significant Difference test as this does not account for multiple comparisons. I also query the use of the Bonferroni-protected contrast because, as I understand it, this should only be used following a significant ANOVA result.

Discussion

Study limitations should be explored. For example, the use of only reflex behaviours to measure pain-related behaviours.

The paragraph outlining the implications to the 3Rs should be changed as I do not believe that this model is a refinement of the use of animals in research. This paragraph should also be moved from the conclusions section. Although, it should be noted that in the future if it does provide a more reliable model of human neuropathic pain then it has the potential to reduce the number of animals used in models that are not clinically relevant.

References

1. Lang T, Altman D: Basic statistical reporting for articles published in clinical medical journals: the SAMPL Guidelines. *Science Editors' Handbook, European Association of Science Editors*. 2013.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 12 Apr 2017

Mary Hannaman, NeuroDigm Corporation, Colorado Springs, USA

Your perspective is appreciated and your concerns have been addressed. EPO pilot study power statistics have been added to the revision. The initial sample size was based on prior investigations.

The Supplemental S3 refinement chart we added depicts how the refinement of a scientific procedure (as referred to by NC3Rs) that limits tissue damage can reduce acute pain, eliminate paw dragging, limb deformities, and self-mutilation. If rodent refinements reduced or eliminated pain their potential as models could be lost.

Your pertinent question about the type of pain the model represents is best answered simply. We strived to elicit the types of evoked pain behaviors referred to as allodynia and hyperalgesia (1) that may develop gradually in patients after soft tissue trauma.

We were trying to mimic in this study the chronic pain sustained for years as seen in humans. Most investigations would not need to be months long and studies can be shortened to any time after post procedure day 23. However, the longer possible duration of this study can possibly reduce the number of animals used in future studies.

There is no homogeneous "sham group" represented by the data that could be used as a control over time. After 3 months 5/8 shams developed pain behavior and there was no single sham animal that had intermediate behavior in the values represented by the graphs. We took the unusual step of presenting the sham data individually to be perfectly clear

about what happened in that interesting group. We purposely did not include asterisks in Figure 5, because it would be misleading. The shams' individual data in Figure 6 resembles the human response — not all humans get neuropathic pain after a soft tissue injury. The EPO pilot study also helps characterize the GEL model by showing the analgesic effect of a localized biologic, which has not been demonstrated in the current models.

The second author (DAF) consulted on experimental design and data display and conducted the statistical analysis of the behavioral data. Aside from expertise in neurobiology and IACUC regulations, he has taught undergraduate statistics and published several articles (2— 6) on experimental design, simulation, and ANOVA.

Regarding the standard deviation instead of standard error, this recommendation by Lang and Altman (7) is curious because they offer no rationale for it in their paper. Use of standard deviations allows estimation of standardized effect sizes, whereas use of standard errors allows estimation of inference (null hypothesis test or confidence interval). Those authors might have had in mind that the use of standard errors without indication of corresponding sample sizes would rule out the estimation of standardized effect sizes by subsequent readers or meta-analyses. That is not the case in our paper because we explicitly include estimates of standardized effect sizes in Table 1. Thus, there is no reason to prefer standard deviations over standard errors, and the latter assist in informal estimation of significance for the many contrasts that we did not explicitly test.

Fisher's protected least significant differences (PLSD) and Bonferroni: Note that planned comparisons can always be tested, unlike post-hoc comparisons (data snooping). We tested only planned comparisons in this paper. We quote the recommendations of experts Milliken and Johnson (8):

1. Conduct an F-test for equal means.
2. If the F-statistic is significant at the 5% level, make any planned comparisons you wish to make by using the LSD method. This includes not only comparisons between pairs of means but also comparisons based on any selected contrasts of the μ_i 's. If one has equal sample sizes, the Waller-Duncan method can also be used. For data snooping and unplanned comparisons, use Scheffe's method.
3. If the F-statistic for equal means is not significant, the experimenter should still consider any individual comparisons that he or she had planned, but should do so using either the multivariate t-distribution method or Bonferroni's method. The experimenter should not do any data snooping in this case. Since the F-test for equal means is nonsignificant, Scheffe's procedure would not yield any significant differences anyway.

Your insightful question "Do the analgesic responses reflect human responses?" highlights a crucial issue that cannot be accurately answered despite extensive discussion in the literature. Presently "pain" in patients and experimental rodents is assessed by different assays. With similar "pain assays" the validity of such translational comparisons can be improved.

References

1. Baron R. Mechanisms of disease: neuropathic pain—a clinical perspective. *Nature clinical practice Neurology*. 2006 Feb 1;2(2):95-106.
2. Fitts DA. Misuse of ANOVA with cumulative intakes. *Appetite*. 2006 Jan 31;46(1):100-2.
3. Fitts DA. Improved stopping rules for the design of efficient small-sample experiments in biomedical and biobehavioral research. *Behavior research methods*. 2010 Feb 1;42(1):3-22.
4. Fitts DA. The variable-criteria sequential stopping rule: generality to unequal sample sizes, unequal variances, or to large ANOVAs. *Behavior research methods*. 2010 Nov 1;42(4):918-29.
5. Fitts DA. Ethics and animal numbers: informal analyses, uncertain sample sizes, inefficient replications, and type I errors. *Journal of the American Association for Laboratory Animal Science*. 2011 Jul 15;50(4):445-53.
6. Fitts DA. Minimizing animal numbers: the variable-criteria sequential stopping rule. *Comparative medicine*. 2011 Jun 15;61(3):206-18.
7. Lang T, Altman D: Basic statistical reporting for articles published in clinical medical journals: the SAMPL Guidelines. *Science Editors' Handbook, European Association of Science Editors*. 2013.
8. Milliken GA, Johnson DE. Analysis of messy data, Volume I: Designed experiments. Wadsworth. Inc. Belmont, California. 1984.

Competing Interests: Author is a principal of NeuroDigm Corporation.

Reviewer Report 14 November 2016

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Michael Brines

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This manuscript describes an interesting rodent model of chronic pain. The basic premise is that local injection of a mixture of biological materials typical of the extracellular matrix following tissue injury activates tissue repair processes which ultimately causes a constrictive nerve injury

associated with the development of pain behaviors. However, the view that traumatic injury is not involved in the development of this model must be incorrect, as the sham animals also develop pain behaviors, albeit to a lesser degree and with a delay compared to the active procedure arm. The biological processes involved are well schematized in Figure S1 and underscores that the involvement of both injury and repair processes in the development of the neuropathic state.

Unfortunately, pathological evaluation was performed only months after the initiating lesion, long after the acute and subacute effects of the procedure had resolved. If the assumption that purely repair processes are involved in this model, it would be necessary to evaluate in a longitudinal manner molecular and other markers of tissue damage and repair. For example, the presence or absence of inflammatory cells and quantification of pro-inflammatory cytokines at the site of injection of the sciatic nerve in the days and weeks following the experimental procedure would determine to what extent inflammation is involved. A better description of the pathophysiology of this model would be that of a delayed neuropathic state developing after mild peripheral nerve trauma.

In terms of general issues, the behavioral methodological details are unusually detailed and to aid readability of the manuscript, much could be moved to Supplemental Materials. The authors are to be congratulated in following very rigorous blinding procedures for the behavioral testing which often are lacking in the published descriptions of neuropathy models. These strengthen greatly the behavioral observations. A particularly interesting observation was the effect of estrogen-containing cream present on the experimenter on the pain behavior observed and further underscores the need for great care in limiting potentially confounding variables which reduce the fidelity of experimental observations.

In spite of the detailed description of the experimental procedures, one question arises: do the authors believe that the injection site targets only the tibial nerve? If so, the exact testing location of the plantar surface will be important, i.e., whether in the tibial or sural nerve distribution. Many peripheral nerve models are characterized by sprouting from the adjacent sural distribution and this possibility needs to be evaluated.

Additionally, there is a redundant exposition of experimental results: as just 2 examples, Figures 2 and 5 present the same data (Figure 2 is perhaps more useful for the reader) as do Figures 5-6. Streamlining the text and exposition of data, i.e. methods and results, would make the manuscript more readable. The "Summary of pain behavior" and "Conclusion" are redundant in my view.

The conclusion in the discussion that this model is one of focal peripheral nerve injury is premature, as evaluation of the spinal cord and dorsal root ganglia was not undertaken. The presence of contralateral behavioral findings, even within the sham group, implies that there is at the least critical spinal cord involvement in this pain model. The authors correctly point out that additional studies focusing on the central nervous system are needed to more completely define the pathophysiology of this model.

The inclusion of EPO-mediated pain improvement raises more questions than it answers and appears almost as an afterthought. Unfortunately, there is not enough detail, especially at the pathological level, to confirm the effect is via "activation of repair". The extremely fast response, occurring over a few days evidently, is not consistent with true repair which requires a much longer timespan. Additionally, the differentiation between local and systemic effects of EPO assumed in this study may not be correct. The dose of EPO administered (400IU/kg) is at the lower

limit needed to exhibit beneficial effects in other neurological models and further, administration via the SC route would reduce the peak plasma EPO concentration. An intraperitoneal or intravenous route would have been preferable. Perhaps this observation would be better moved to supplementary material and speculation on the underlying biology limited.

In conclusion, this model is interesting and the very detailed methodology makes it a useful addition to the study of pharmacological treatment of pain behavior. The clear distinction between classes of agents used as treatment for neuropathic symptom increase optimism that the model can be useful for screening potential efficacy of new compounds or evaluating novel methods of treatment using existing pharmacotherapy.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 12 Apr 2017

Mary Hannaman, NeuroDigm Corporation, Colorado Springs, USA

The value of your knowledge of tissue repair is useful in understanding the hypothesis of our model. We realize our focus on the later stages of our model presents frustrations. For this study we merely wanted to develop and then treat the chronic pain behavior as seen in patients. Once established as being representative of patients, earlier studies can be done. Also the extensive testing limited the number of rodents able to be used for specimens in this lengthy study.

The paper's main tissue focus is on the neural histology findings long after pain behaviors are established, limiting any explanation of the exact pathophysiology. The hydrogel does not directly cause traumatic injury or acute inflammation in the first 14 days, as evidenced by the lack of significant pain behaviors, erythema, cyanosis, edema, or altered gait. Interestingly, the shams that developed late onset pain behaviors had no evidence of tissue damage on light microscopy, and may represent an obscure neural "dysfunction".

Regarding the redundant exposition of experimental results: We agree that Figure 2 is in many ways more informative, and we used Figure 5 to provide a formal statistical analysis of the null hypothesis. Omitting Figure 5 would omit the statistical analysis.

We agree that EPO does not likely alleviate the pain through tissue repair. The erythropoietin's neuroprotective mechanisms are not known for this neural model and need further study.

The EPO dose in mg/kg units is close to 300 units/kg. The systemic EPO dose was subcutaneous to limit stress, tissue damage and complications. The repeat injection of EPO in 2/5 GEL rats discussed in paper suggests localized EPO placement is critical for this low dose to cause a reversal effect.

You are correct in stating that this model of focal neuritis creating neuropathic pain may involve more than the discrete site on the nerve. The spinal cord and brain are likely involved, and we saved the brains, as well as non-neural tissue, of the rats for further study.

Your other concerns have been incorporated in the revision.

Competing Interests: Author is a principal of NeuroDigm Corporation.

Reviewer Report 26 October 2016

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Odd-Geir Berge

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This article presents a novel model of neuropathy with slow onset of symptoms (increased responsiveness to mechanical stimuli) that, once established, persist throughout the study period of several months. Another feature of the model is the use of adult rats. The model is characterized by means of some commonly used drugs, histology, and, as a pilot study added after conclusion of the main study, administration of erythropoietin to a subgroup of animals.

While the experimental procedures as such appear to be technically solid, reservations may be raised concerning terminology, presentation of background including literature references, translational context, discussion, and validity of conclusions. The structure of the paper can be improved by prioritizing and moving content to relevant sections, which would facilitate reading and increase the focus on important findings. In addition, the erythropoietin “pilot study” was carried out under less stringent conditions and may perhaps be reported as a separate short or preliminary paper.

Major concerns:

The authors equate responses evoked by mild mechanical stimuli (von Frey stimulation, gentle brushing, pin prick) with pain behavior and clinical pain. Even terms like allodynia and hyperalgesia are used to describe these responses although the paper provides no evidence that there is any pain involved in the model or that these responses are valid surrogate markers for clinically relevant pain. These types of stimuli are undoubtedly useful both in psychophysical studies and quantitative sensory testing but rarely used to evaluate analgesic efficacy in clinical trials. I would suggest using neutral, descriptive terms throughout the manuscript and bring up the translational relevance in the Discussion, taking into account the extensive literature on this topic.

The pharmacology is interpreted in terms of analgesia, which is questionable since there is no evidence for pain in the model. The drugs used, perhaps with the exception of celecoxib in low doses, have known non-analgesic effects that may interfere with evoked responses. While pharmacological characterization of a model is useful for various reasons, clinical relevance is a different matter and clinical efficacy is generally poorly predicted by animal models of neuropathy. This is arguably the case here where the efficacy of the tested drugs are much more robust than the case is in well-controlled clinical trials and the order of efficacy, in spite of what is claimed, is at variance with the clinical data (for a recent, comprehensive review, see Finnerup et al¹). It should be pointed out that comparing drug efficacy in single-dose experiments has limited utility, especially when pharmacokinetic data are lacking. Relating drug doses in this animal study to human clinical data is of course relevant but should be done in the Discussion with due reference to the many limitations of the current approach.

The use of literature references is in part inadequate. For instance, the short first paragraph of the introduction contains no less than 30 references and it is hard to identify the ones that may have relevance for the statements in the text. Many of these references deal with conditions that are connected to the topic of the present study only in a general sense and it is not obvious how they may support the arguments. Since delayed development of sensory changes seems to be a main feature of the described model, specific reference to one or two key clinical papers would be helpful. It may be necessary to carefully match the text of the manuscript with the references and perhaps delete sections or statements that are not supported by literature; alternatively to reorganize the text so that speculations and personal opinions are clearly differentiated from information supported by evidence in the literature.

Specific suggestions & comments

- Title (and in other places):
The described method is referred to as “refined” but compared to what is not defined in the manuscript.
- Abstract:
Background: The paragraph should be refocused to state the objectives / hypotheses of the study.

Methods: The first sentence belongs to the Results paragraph. Mention test methods and readouts. Some details e.g. age of rats, doses, routes of administration of drugs, would give the reader a chance of understanding what to expect from the paper. Generic drug names should not be capitalized (correct in the following section).

Results: As in the rest of the manuscript, avoid controversial terms like “pain behavior” and “analgesia”.

Conclusion: The text is inaccurate in suggesting that there is direct evidence for pain behavior in the model and that this behavior is related to remodeling; the difference between the sham and gel treatments is mostly quantitative allowing for other interpretations. It is not obvious that the effects of analgesics in the model reflect clinical efficacy.

- Introduction:

In addition to the more general points raised above, concentrating the text on the present model into a single paragraph with a clear statement of objective may facilitate reading and sharpen focus. The text regarding relevance of animal models do not adequately address the rather extensive discussion that has been ongoing at meetings and in the literature for quite a while and the argument put forward is unspecific. There is room for a more complete treatment in the Discussion where a number of issues relevant to the present study could be addressed, e.g. interpretation of readouts and the importance of pharmacokinetic factors. In the introduction, perhaps pointing to the expected advantages of the present paradigm compared to previous work would suffice.

- Materials and methods:

Bodyweight of animals also at the time of testing would be interesting.

P5, left column, 4th paragraph: How was the forces of the von Frey filaments confirmed? Are these filaments stable under changing environmental conditions?

P5, last paragraph: the meaning of the phrase "all with no change in pain behavior noted" is unclear.

Some text could be deleted or moved to the discussion to provide easier reading, e.g. p5, left column, 3rd paragraph ("Measures of ..."), right column 2nd paragraph ("The original doses ...")

- Results:

In general, this part of the manuscript would benefit from limiting the text to what is necessary to understand the data, leaving discussion to the Discussion. Even figure legends should be edited to remove interpretations, speculations and explanation of what can be easily seen from the graphs. All symbols should be explained in the legends. Number of animals, indication of statistical analysis (with details in methods and results), and how the means are calculated could be given in the legends and thus allow for a more succinct main text.

P7 & 8: "Results of behavioral data" - the first four paragraphs should be deleted or condensed and moved to the discussion (with appropriate succinct information added to the figure legends).

The following paragraphs "Days of data..." could be condensed to a few descriptive sentences and the justification for various procedures moved to the discussion.

I would suggest that Table 1 and associated text be moved to Supplemental material or deleted altogether; minor changes in the results may change the numbers significantly so the value of these calculations for future studies would be too limited to warrant an extensive presentation as in the present manuscript.

P9, Section on von Frey: For consistency: mention the results of the GEL group. The sentence "Asterisks ..." should be moved to the figure legend (this also applies to the following sections).

P10: "Individual data...": It is difficult to see what this analysis contributes to the paper – in my view it distracts from the main findings and should be deleted or moved to Supplemental material.

The following paragraph "Summary ..." is redundant; the "Factor influencing ..." is anecdotal and probably best deleted, alternatively mentioned in the Discussion.

"Results of experiments...": the information in the first paragraph fits better in other parts of the manuscript.

Section on morphine: the first sentence is redundant / irrelevant.

Later in the paragraph (p 11), the explanation of asterisks can be removed twice, it's adequately explained in the figure legend (same applies to text later in the Results).

The last paragraph on morphine does not appear to add value.

p14: "Summary..." is redundant.

- Discussion:

I would recommend an extensive revision of the Discussion, addressing methodological aspects and taking into account the limitations of the approach, some of which are delineated above. Careful scrutiny of the references to make sure that they are fair and representative as well as addressing the statements in the text would be another recommendation, also pertaining to the rest of the manuscript. As indicated above, there may be material in previous sections of the manuscript that would fit in the Discussion.

- Conclusion:

Appears more as a continuation of the discussion and should be revised, preferably to reflect the objectives of the study and the initial hypotheses.

References

1. Finnerup NB, Attal N, Haroutounian S, McNicol E, et al.: Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. *Lancet Neurol.* 2015; **14** (2): 162-73 [PubMed Abstract](#) | [Publisher Full Text](#)

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 12 Apr 2017

Mary Hannaman, NeuroDigm Corporation, Colorado Springs, USA

The in-depth analysis you provided was carefully examined and applied. Some areas were retained based on the comments of another, as in Table 1, which has the standard deviations desired by some. In this lengthy research report, discussions of translational context and pharmacokinetics are not covered.

The references have been abridged for relevancy, as suggested. The hypothesis and

objective have been included in the Abstract and Conclusion. Paragraphs have been moved to more relevant headings. Summaries were deleted. Clarifications regarding refinement are discussed in the revision (Supplement S3).

Early recognition of a hormonal influence on behavioral inconsistencies was crucial to the study's completion. If the role of estrogen had not been realized the study would have been terminated.

The very thorough paper by Finnerup et al 2015 "Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis" (1) classifies gabapentin and duloxetine as first-line drugs for neuropathic pain with a "high quality of evidence" for both, and strong opioids as a third-line choice. These analgesic classifications are supported by the findings in our study.

We agree that "clinical efficacy is generally poorly predicted by animal models of neuropathy" since their pathophysiology does not reflect what usually happens in patients. We also consider the traditional neuropathic pain assays of mechanical hypersensitivity used for decades in research labs, that we used, to have unrecognized translational merit — as you note they are "rarely used to evaluate analgesic efficacy in clinical trials", but should be considered in our estimation.

As suggested, an explanation of the validity of the conclusions has been added to the revision in the Discussion.

References

1. Finnerup NB, Attal N, Haroutounian S, McNicol E, Baron R, Dworkin RH, Gilron I, Haanpää M, Hansson P, Jensen TS, Kamerman PR. Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. *The Lancet Neurology*. 2015 Feb 28;14(2):162-73.

Competing Interests: Author is a principal of NeuroDigm Corporation.

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