

# 1 **Tibial Nerve Transection (TNT) Model of Neuropathic Pain in the Rat**

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## 11 **Abstract/Summary**

12 Neuropathic pain is a highly prevalent condition worldwide, with a range of complex aetiologies  
13 related to nervous system damage. There are a number of animal models which are used to mimic  
14 clinical symptoms observed in neuropathic pain patients, including nerve injury and drug/tumour-  
15 induced neuropathies. The model described is one of several peripheral nerve injury models used in  
16 the rodent to mimic clinical symptoms of neuropathy, involving transection of the tibial nerve. This  
17 protocol describes the surgical procedure carried out to induce this injury, alongside a standardised  
18 method to assess development of pain responses, measured by mechanical hypersensitivity, over a  
19 period of weeks post-surgery. This model represents a surgically un-complicated model,  
20 approximately 5- 10 minutes per animal, which produces a robust and long lasting hypersensitivity,  
21 present up to 80 days post-surgery. This consistency allows the assessment of potential analgesic  
22 agents at a range of times post-surgery, which could be correlated with changing pathological  
23 conditions.

## 25 **Introduction**

26 Despite the widespread prevalence of neuropathic pain<sup>1</sup> there are currently few effective analgesics  
27 for the treatment of this type of pain approved, and with causes as wide ranging as surgical  
28 procedures, diabetes-induced neuropathy, HIV and anti-retroviral drugs, chemotherapeutic agents,  
29 metabolic disease and traumatic injury it is clear why this condition is hard to treat. Diagnosis of

1 neuropathic pain in patients is based on the presence of persistent abnormal responses to painful  
2 (hypersensitivity) and normally non-painful stimuli (allodynia) or spontaneous pain, and can be  
3 classified generally into peripheral or centrally initiated neuropathies <sup>2</sup>. Several sub-sets of pain  
4 response can be characterised in the clinical population experiencing neuropathic pain, including  
5 patients displaying mechanically-induced static (von Frey filament stimulation) or dynamic  
6 hypersensitivity (brush stimulation), cold hypersensitivity, mechanically-induced hypersensitivity  
7 (paw pressure or compression), and spontaneous pain <sup>3,4</sup>. Thus, it seems appropriate to consider the  
8 use of a variety of in vivo animal experimental models to screen for potentially clinically useful  
9 analgesic compounds, dependent on the clinical target population. To this end there are a wide  
10 variety of animal models currently used, with the nature of the injury reflecting the variety and type  
11 of aetiologies observed in patients. These include HIV-induced neuropathy models <sup>5</sup>, cancer-induced  
12 models <sup>6</sup>, drug-induced models <sup>7,8</sup> and surgical-induced damage to the central and/or peripheral  
13 nervous system <sup>9-12</sup>.

14

### 15 **Peripheral Nerve Injury Models**

16 Clinically, peripheral neuropathies represent a large proportion of patients suffering with  
17 neuropathic pain <sup>13</sup>. There are currently several peripheral nerve injury models used in the rodent to  
18 help predict analgesic efficacy in these patients, and indeed, to further understanding of the  
19 underlying pathophysiological mechanisms behind these conditions. Although peripheral nerve  
20 injury is commonly induced by trauma in rodent models, this is only one of the possible causes of  
21 clinical peripheral neuropathies. The rodent models involve surgical procedures and can be  
22 categorised based on the location and nature of the traumatic injury involved (Figure 1), which may  
23 lead to subtly different pathophysiological and behavioural changes. Initially axotomy models were  
24 used to induce neuropathic pain, however, this model also caused autotomy which could be  
25 considered of limited relevance to the human conditions observed <sup>14</sup>. Currently the commonly used  
26 models of neuropathic pain in rodents include chronic constriction injury (CCI), partial sciatic nerve  
27 ligation (PSL), spinal nerve ligation (SNL) (reviewed in <sup>15</sup>) and spared nerve injury (SNI) models<sup>18</sup>. Of  
28 these, only the SNI model involves transection of a peripheral nerve, whereas, the CCI, PSL and SNL  
29 involve differing degrees of ligation of peripheral nerves.

30

31 The CCI model first described by Bennett and Xie in rats <sup>16</sup> involves dissection through the biceps  
32 femoris to expose the common sciatic nerve, with four loose ligatures (4-0 chromic gut or silk

1 sutures) then placed around the sciatic nerve, as illustrated (Figure 1). PSL and SNL surgery are  
2 broadly similar to this, with either the sciatic nerve or L4-6 spinal nerves visualised before partial  
3 ligation of the sciatic nerve or full ligation of L5 and L6 spinal nerves respectively, carried out with  
4 silk sutures<sup>17 18</sup>. A major advantage of these ligation models over earlier sciatic nerve axotomy  
5 models is that nerve innervations to the hindpaw are not abolished, thus producing hypersensitivity  
6 in the hindpaw without complete loss of sensation, which increases the risk of autotomy.  
7 Alternatively SNI surgery differs in the nature and location of the injury. This surgery involves  
8 visualising the sciatic nerve distal to the spinal cord before tightly ligation of the common peroneal  
9 and tibial branches of the sciatic nerve are performed (Figure 1). The nerve is then transected distal  
10 to the ligation and a portion of the nerve stump removed<sup>19</sup>, still allowing nerve innervations to the  
11 hindpaw through the sural nerve.

12 Following all three types of peripheral nerve injury involving ligation of a peripheral nerve (CCI, PSL  
13 and SNL) animals display mechanical hypersensitivity, thermal hyperalgesia and spontaneous pain  
14 behaviours, although to differing degrees and following different time courses<sup>15</sup>. Typically,  
15 however, the window in pain response is lost approximately 6-10 weeks post-surgery following  
16 these procedures<sup>(18,20)</sup>, and due to variability in ligation technique, for example how tightly the  
17 surgeon ties the ligatures, the exact location, type of knot/suture used there is the possibility of  
18 greater variability in results. By contrast, despite the clear difference between ligation and  
19 transection in terms of damage to nerve, SNI surgery also produces hypersensitivity and  
20 spontaneous pain, although more robust and long-lasting<sup>19,20</sup>.

21 Following induction of hypersensitivity in these models animals show similar pharmacological  
22 sensitivity to clinically used analgesic compounds such as morphine, mexiletine and gabapentin<sup>21-24</sup>.  
23 It has been suggested that there are subtle differences in pharmacological sensitivity in the SNI  
24 model compared to other ligation models<sup>22</sup>, however, these changes are often dependent on the  
25 pain end point measured, and the time post-surgery.

26

### 27 **Tibial Nerve Transection (TNT) Model**

28 Within the last decade transection of the sciatic nerve branches; tibial, common peroneal and sural  
29 has been considered in more detail<sup>25</sup>. These three nerves innervate the hindpaw, and thus  
30 cessation of nerve transmission from one, or more, of these nerves leads to the development of  
31 hypersensitivity in the regions innervated by the remaining nerves<sup>25</sup>. Lee et al., 2000 demonstrated  
32 the effects of transection of the different combinations of the three nerves on pain responses in the

1 rat, and suggested that the result of this injury appears to be more sympathetically independent  
2 compared to models such as CCI, PSL and SNL.<sup>25</sup> Of the possible combinations, tibial and sural nerve  
3 transection, or tibial nerve transection (TNT) alone, produced the most robust change in pain  
4 threshold, with a greater magnitude of response to von Frey stimulation observed following either  
5 combination of transection compared to SNI surgery (leaving only sural nerve intact). In addition the  
6 TNT model involves comparably less damage to tissues due to the relative simplicity of the surgery  
7 than ligation models, which may lead to a less marked inflammatory response as suggested by the  
8 apparent lesser role of central COX2 in the SNI model compared to SNL<sup>26,27</sup>. The TNT model  
9 described produces a long lasting and robust mechanical hypersensitivity, which compares more  
10 favourably with clinical symptoms, in terms of chronicity of the model, than ligation models. This  
11 allows pharmacology to be investigated over a longer time period post-surgery, at more advanced  
12 stages of the response to injury, during the maintenance of neuropathic pain. Similar to the SNI  
13 model, and ligation models, clinically effective analgesic compounds display efficacy in TNT animals  
14 using a mechanical hypersensitivity end point<sup>28</sup>, thus demonstrating the potential to translate to the  
15 clinic.

16

17 TNT, overall, represents a surgically uncomplicated model which produces robust hypersensitivity  
18 still present 10 weeks post-surgery, with a region of hypersensitivity on the medial and lateral  
19 aspects of the ipsilateral hindpaw. The major advantages associated with this protocol compared to  
20 other common models is the relative simplicity and speed of the surgical procedure coupled to the  
21 ability to assess pain development and maintenance chronically, and with more consistent results  
22 due to the definite transection of the nerve rather than potentially surgeon-dependent differences  
23 in ligation technique. Within this protocol TNT surgery is described in the rat, with the effect of this  
24 surgical procedure upon mechanical hypersensitivity used as a representative reflex-mediated pain  
25 response, measured using von Frey hair stimulations with a modified up-down method of testing to  
26 determine the 50% paw withdrawal threshold (PWT)<sup>29</sup>. This protocol demonstrates a standard  
27 course of training and testing used to measure 50% PWT, and thus assess levels of mechanical  
28 hypersensitivity pre and post-treatment as an indicator of analgesic efficacy, in established  
29 neuropathic pain. However, as already mentioned, there are numerous types of responses to  
30 mechanical and thermal stimuli observed post-TNT surgery which allows this procedure to be  
31 adapted in order to investigate alternative mechanisms, end-points, and different species, such as  
32 mouse. The level of detail described should permit close reproducibility of this method between  
33 laboratories, and allow comparison of this method with other neuropathic pain models.

1

## 2 **Experimental Design**

### 3 **TNT Procedure**

4 The TNT surgical procedure described herein comprises of a loss of tibial nerve innervations to the  
5 ipsilateral hindpaw, by tight ligation of the tibial nerve in two places prior to transection between  
6 ligations. Following TNT surgery typically 100% of TNT rats will display decreased pain thresholds in  
7 our hands, which is consistent with previous literature<sup>28</sup>, and the transection ensures results are not  
8 as surgeon-dependent as ligation models. However, clearly this procedure still requires specialised  
9 surgeons to carry out the procedure quickly and effectively, with several influential stages. In  
10 particular, we feel that gaining access to the tibial nerve by separating the biceps femoris muscle, as  
11 opposed to cutting through the muscle (carried out in several other ligation and transection models),  
12 aids comfort and recovery for the animal and helps improve access to the nerve. In addition,  
13 placement and tightness of sutures, especially skin wound clips can also influence behaviours  
14 observed post-surgery.

### 15 **Controls**

16 Following TNT surgery a robust mechanical hypersensitivity is observed in the ipsilateral hindpaw  
17 indicated by a decrease in 50% PWT, which is measured most clearly on the lateral portion of the  
18 hindpaw, with no change in response threshold in the contralateral hind paw and comparable to  
19 naive rats. This effect is consistent throughout the time-course post surgery, which allows the use of  
20 contralateral values as an internal control within a study. Despite 50% PWTs in contralateral  
21 hindpaw being similar to naive rats it may also be necessary, dependent on the study design, to  
22 include a naive group to demonstrate full reversal of TNT-induced hypersensitivity. Often it is also  
23 appropriate to include sham-operated rats within studies in order to isolate the neuropathic pain  
24 development component from any effects of surgery alone. The surgery to produce sham rats is  
25 exactly the same as the TNT procedure, with the exception that once the nerve is visualised no  
26 ligation or transection is performed, and the wound closed immediately after. In our experience, we  
27 commonly observe a degree of hypersensitivity in sham-operated rats immediately post-surgery,  
28 which resolves by approximately 1 week post-surgery, suggestive of a post-surgical, but not chronic  
29 neuropathic pain. Thus, in studies conducted within the first week after TNT surgery it is important  
30 to recognise that sham-operated rats may not represent a pain free control. When deciding on  
31 appropriate controls in fully blinded studies with TNT and sham-operated rats it is crucial to realise

1 that TNT rats consistently hold their ipsilateral hindpaw with a slightly different posture to naive or  
2 sham-operated rats when walking. The lateral aspect of the hindpaw is elevated slightly with  
3 pressure placed through the medial aspect, rather than flat on the plantar surface, which makes it  
4 difficult for experimenters to be blind to surgical state. Therefore, in every study, regardless of  
5 inclusion of naive or sham-operated rats, vehicle-treated rats should always be included and  
6 compared statistically with any treatment.

7

## 8 **Assessment of mechanical hypersensitivity**

9 The mechanical hypersensitivity outcome measure is a commonly used method of assessing surgery-  
10 induced pain responses, detecting threshold responses to punctuate stimuli, von Frey filaments,  
11 which are also used clinically<sup>30</sup>. Initially following surgery general behaviour and weight of the  
12 animals are monitored, with wound clips removed 7 days post-surgery, to ensure sufficient wound  
13 healing, and in accordance with welfare guidelines. Subsequently, 14 days post-surgery rats are  
14 habituated to testing arenas for approximately 45 mins, followed by sequential application of a 15 g  
15 and 8 g von Frey filament to each hindpaw (described fully in methodology section). On the two  
16 days following this rats are again habituated to arenas with application of 8 g and 4 g filaments on  
17 the second days of training, and 4 g then full up-down method of testing to measure 50 % PWT<sup>29</sup>.  
18 Details of the up-down method as described in more detail in the methodology section, in brief, this  
19 consists of sequential application of von Frey filaments over a range from 0.4 – 15 g to determine  
20 the pattern of responses around the threshold response to calculate a 50% PWT value. This 14 day  
21 time point was chosen based on previous evidence demonstrating robust effects of surgery on  
22 mechanical hypersensitivity from 14 days post-surgery<sup>28</sup>, which is consistent with results in our  
23 studies, however, rats can also be trained earlier or prior to surgery. Typically, we observe higher  
24 variability in the first full up-down test compared to subsequent tests, hence the inclusion of this in  
25 the training period. Testing arenas consist of perspex boxes (~20 cm<sup>3</sup>) placed on a row of flooring  
26 consisting of thin metal bars running horizontally approximately 1 cm apart, this flooring is elevated  
27 on racks to allow access to the rat's hindpaws between the bars from underneath. Application of  
28 the von Frey filaments is carried out to the lateral portion of the hindpaw approximately level with  
29 pads around the base of the toes, but not on the toes of the rat. In our experience the up-down  
30 method described by Chaplan et al., provides reliable, robust responses which, due to the  
31 statistically designed calculation of 50% PWT, provide a more continuous-type of data than standard  
32 threshold values (i.e. values obtained are still of limited number, but range between the discrete

1 values on the filaments). In cases where behavioural measurements need to be investigated prior to  
2 14 days post-surgery, at the development of hypersensitivity stage, an adapted version of this  
3 training and testing can be used, for example, prior to TNT surgery rats habituation and baseline 50%  
4 PWT values can be obtained, pain measures can be taken from day 1 post-surgery. Inclusion and  
5 exclusion criteria are set based on previous data obtained using this method, with animals included  
6 in a study if 50% PWT measures are <3 g in the ipsilateral hindpaw and > 8 g 50% PWT in  
7 contralateral hindpaw. It is possible to include animals in a study if values obtained are close to  
8 these criteria, typically no more than 2 rats from 8, still maintaining a window between ipsilateral  
9 and contralateral/naive values.

10

## 11 **Study Design**

12 Crucial to the interpretation of TNT studies is the confidence that each study has been completed in  
13 an unbiased and balanced manner. These issues are particularly important in a behavioural study  
14 which has some element of subjective assessment, and is now increasingly recognised by both  
15 industry and academic researchers, as gold standard not only for clinical studies<sup>31</sup>, but also within  
16 pre-clinical study design<sup>32</sup>. In order to ensure a balanced study rats are randomised to treatment  
17 groups based on their baseline ipsilateral 50% PWT scores manually by a second experimenter, on  
18 each individual day of testing (could also be carried out using random number generators). This  
19 ensures equal spread of treatments on any given period of testing and similar baseline scores across  
20 groups to assess changes against. In addition to this randomisation all studies are performed fully-  
21 blind, with a second experimenter carrying out randomisation and dosing of animals to ensure the  
22 experimenter is unaware of treatment groups. This is in contrast to a single-blind study where the  
23 experimenter doses coded treatments and is only blind to treatment code. Blinding studies in this  
24 manner adds a further level of confidence that the study has not been influenced by bias. We find,  
25 following statistical analysis of initial studies (2-sided, 5% significance) that 6-8 animals per  
26 treatment group provides 80% power to detect a difference from 4.1 to 4.9 g between vehicle and  
27 compound treated animals.

28

## 29 **Other behavioural pain outcome measures**

30 As previously discussed, TNT surgery causes a range of altered pain responses which can be assessed  
31 in the rat and we have found that alternative end-points to mechanical hypersensitivity, such as cold

1 hypersensitivity, can follow different time courses. Measuring 50% PWT may also be useful as a  
2 technical positive control alongside the investigation or development of other end-points, to confirm  
3 that TNT surgery has been successfully carried out.

4

## 5 **Materials**

6

## 7 **Reagents**

8 • Male pathogen-free Sprague-Dawley rats (approx 300 -350 g at time of surgery) (Charles  
9 River, UK) **!CAUTION** All experiments must comply with relevant local regulations regarding  
10 use of animals in research **▲ CRITICAL** Changes to strain, weight and gender of rats may  
11 affect outcome measures.

12 • Isoflurane (e.g., Centaur services, cat no. 30135687)

13 • Lacrilube (e.g., Centaur services, cat no. 21190480)

14 • High level disinfectant (e.g., Trigene Advance, Nu-care, cat no. TR099)

15 • Hydrex solution (Surgeon's hand and arm scrub) (e.g., Nu-care, cat no. M8001)

16 • Iodine scrub solution (e.g., Nu-care, cat no. M7630)

17 • Iodine antiseptic solution (e.g., Nu-care, cat no. M7640)

18 • Betadine Spray (e.g., Nu-care, cat no M7646)

19

## 20 **Equipment**

21 • Surgeon personal protective equipment – gown, mask and gloves (e.g., Nu-care, cat no.  
22 M90016 + D100 + GSN60) **!CAUTION** protective equipment for surgeon to aid sterile nature  
23 of surgery and protect from animal allergens.

24 • Surgery assistant and behavioural experimenter personal protective equipment – mask and  
25 gloves (e.g., Nu –care, cat no. M149 + GN60SM)

26 Surgical preparation:

27 • Anaesthetic chamber, and nose cone



- 1 • Scrubbing brush (e.g., Nu-care, cat no. M1607)
- 2 • Sterilised surgical equipment (excluding sterile sutures, blanket and rat clippers for shaving
- 3 of fur) by autoclave
- 4 • Green buster drape (e.g., Centaur Services, cat no. 03451301)
- 5 Surgical equipment:
- 6 • Rat fur clippers (e.g., Oster Golden A5 two-speed clipper, Model 5-50, Harvard Apparatus,
- 7 cat no. 01210122)
- 8 • Sterile suture vicryl W9834T (for muscle) 4.0 gauge (e.g., Codmen, cat no. – w9834t)
- 9 • Sterile suture mersilk W211 (for ligation) 4.0 gauge (e.g., Codmen, cat no. – w211) ▲
- 10 **CRITICAL** We suggest using new ligation silk for each surgery as, in our experience,
- 11 autoclaving alters physical characteristics of suture and may lead to variation in injury.
- 12 • Scalpel blade and handle (size 15) (e.g., Nu-care, cat no. M030 + M0933)
- 13 • Straight and curved forceps (e.g., Fine science tools, cat no. 11008-13 + 11009-13)
- 14 • Rat tooth forceps (straight) (e.g., Fine science tools, cat no. 11027-12)
- 15 • Stevens tenotomy scissors (e.g., Fine science tools, cat no. 14066-11)
- 16 • Needle holders (e.g., Fine science tools, cat no. 12500-12)
- 17 • Swabs (e.g., Nu-care, cat no. M1861)
- 18 • Cotton buds (e.g., Nu-care, cat no. M986)
- 19 • Homeothermic heat blanket (e.g., Harvard Apparatus, cat no. DC1-50-7214)
- 20 • Wound clip applier, clips and clip remover (e.g., Fine science tools, cat no. 12031-09 +
- 21 12032-09)
- 22 • Microscope (e.g., Zeiss, product code - OPNIMDU)
- 23 • Bead steriliser (e.g., Harvard Apparatus, cat no. DC1 72-0121)
- 24 Assessment of mechanical hypersensitivity:
- 25 • Perspex arenas on raised platform (parallel metal bars) allowing access to plantar surface of
- 26 hindpaw
- 27 • Von Frey monofilaments (0.4, 0.6, 1, 2, 4, 6, 8 & 15 g) (e.g., Bioseb, cat no. NC-17775)
- 28

## 29 **Equipment Setup**

### 30 **Surgical setup**

31 Typically induction of anaesthesia was carried out by a surgery assistant in a non-aseptic area using

32 an anaesthetic chamber, when a surgical plane of anaesthesia is obtained the site of surgery was

1 shaved and cleaned (as described later) prior to moving the rat to the sterile surgical set-up (Figure  
2 2). The surgeon performed surgery using aseptic technique, with the level of anaesthesia  
3 maintained using isoflurane given through a nose cone, with body temperature of the rat  
4 maintained using a homeothermic heated blanket.

5

## 6 **Mechanical hypersensitivity testing**

7 Rats were placed in perspex arenas approximately 20 cm<sup>3</sup> in size, with 12 arenas placed on each rack  
8 (Figure 3). To ensure rats were not distracted by neighbouring rats a perspex wall between rats in  
9 each row of white opaque material was used. Metal bar flooring and the portion of the racking  
10 under the flooring was cleaned with diluted disinfectant (e.g. Trigene Advance) between each test,  
11 perspex walls were not cleaned during experiments as the same rat was placed in the same arena  
12 for each test, thus maintaining individual rat scents within each arena. The behavioural  
13 experimenter sat below the raised arenas and applied von Frey filaments in a sequential order,  
14 based on Chaplan method <sup>29</sup>, described fully in procedure methods. **! CAUTION** It is possible to  
15 adapt this basic setup to improve ergonomic design of setup, e.g., addition of a movable platform to  
16 steady arm or use of a mirrored surface to visualise hindpaw.

17

## 18 **Procedure**

### 19 **Pre-operative Preparation of Animal**

- 20 **1** Identify, weigh and give animals a general health check. **▲ CRITICAL** Habituation of animals to  
21 local housing conditions is carried out for at least 5 days in accordance with welfare regulations,  
22 in groups of 4, on a 12 hour light-dark cycle with access to food and water ad libitum.
- 23 **2** Sterilise all surgical equipment in an autoclave at 134°C for 3minutes and 25 seconds, within a  
24 cotton sheet. Subsequently ensure equipment sterility is maintained using a bead steriliser  
25 between surgeries.

### 26 **Surgical Preparation**

- 27 **3** Induce anaesthesia with inhalative isoflurane (5%) and oxygen (1.5 L/min) in an anaesthetic  
28 chamber to effect. **! CAUTION** Depth of anaesthesia must be sufficient for surgical plane and  
29 monitored throughout the procedure using toe pinch or corneal reflex to prevent suffering to  
30 the animal.

- 1     **4** Apply Lacrilube™ to the cornea to provide ocular protection for the duration of the procedure.
- 2     **5** Shave the right hind limb of the animal at the level of the thigh to midline.
- 3     **6** Next apply iodine scrub solution with a swab to the shaved area, to clean and disinfect the
- 4         surgery site (appropriately diluted), followed by application of iodine anti-septic solution to the
- 5         cleaned area (again, appropriately diluted).
- 6     **7** Transfer the animal to the surgical setup and maintain anaesthesia by inhalation of oxygen (1.5
- 7         L/min) and isoflurane (approximately 2%) through a nose cone for the duration of the surgery.
- 8         Place the rat in ventral recumbency with its head facing away from surgeon, on a
- 9         homeothermic heat blanket. **! CAUTION** Prolonged exposure to isoflurane may produce
- 10         respiratory arrest, monitoring of depth and length of anaesthesia required throughout
- 11         procedure.

12

### 13     **TNT Surgical Procedure**

- 14     **8** Make an incision through the skin approximately 1.5cm in length, over and in line with the femur
- 15         of the right hind leg. **▲ CRITICAL STEP** Incision length may be greater than 1.5 cm in length until
- 16         surgeon gains experience in the technique, and can optimise incision positioning and skin
- 17         opening to perform transection effectively.
  
- 18     **9** Next clear the connective tissue under skin and locate the fascial plane, located posterior to the
- 19         femur, between the gluteus superficialis and biceps femoris muscles (visible white line). **▲**
- 20         **CRITICAL STEP** Making the incision between the muscles, as opposed to through the muscle,
- 21         restricts damage to normal physiology of the animal and also improves ease of access to nerve
- 22         for surgeon.
  
- 23     **10** Holding the biceps femoris muscle taut with rat tooth forceps nick a small incision in the centre
- 24         of the fascial plane identified by white line, using the Stevens tenotomy scissors, using a
- 25         microscope to view the area clearly if necessary. Gently expand this hole then expose the sciatic
- 26         nerve which lies at the bottom between the muscles, a vein running at approximately 45
- 27         degrees across the field should be observed at the right hand side of the dissection.
  
- 28     **11** Using curved forceps gently tease away the nerve from the surrounding connective tissue and
- 29         fascia. Follow the sciatic nerve distally towards the knee gently blunt dissect the fat surrounding
- 30         the nerve. Locate and visually identify the three nerve branches off the sciatic nerve which
- 31         become visible as tibial, sural and common peroneal nerves. The smallest, the sural nerve, can

1 be seen branching off the sciatic nerve first and running caudally, then the common peroneal  
2 branch becomes visible running more laterally into the muscles of the thigh and, finally, the  
3 thickest, the tibial nerve, can be seen running more cranially towards the front of the thigh,  
4 adjacent to the blood vessel identified previously. ▲ **CRITICAL STEP** It is essential to successfully  
5 identify which of the three branches is the tibial nerve, using anatomy descriptions of these  
6 branches as shown in Figure 4.

7 **12** Place the curved forceps, with silk ligature (W211) attached, under the tibial nerve and carefully  
8 ligate the nerve 2-3mm away from where tibial nerve branches from the sciatic nerve as  
9 illustrated in Figure 4. Trim the ends of the suture to leave approximately 2 mm visible. ▲  
10 **CRITICAL:** We found that it was advantageous to use new (not previously used autoclaved silk)  
11 silk as autoclaving appears to fray suture material making ligation intensity more variable,  
12 therefore, using new, non-frayed silk provides more consistent results.

13 **13** Repeat step 12 approximately 2mm further down the tibial nerve caudally, prior to the  
14 subsequent branching of the nerve.

15 **14** Following completion of these tight ligations transect the tibial nerve between the two ligatures  
16 using Stevens tenotomy (or vanna scissors). Subsequently, gently place the nerve into its correct  
17 anatomical position and close the muscle back over it with a single suture using vicryl suture  
18 thread (W9834T), ensuring the silk attached to the nerve does not become interwoven with this  
19 suture. ▲ **CRITICAL STEP** Ensure transection of tibial nerve is clean, with no unintentional  
20 damage to surrounding tissue, which may lead to unanticipated effects of surgery.

21 **15** Close the skin incision with metal wound clips, by placing the two skin edges together and  
22 holding taut whilst applying wound clip applicator to the skin. We observe that 2-3 clips per  
23 animal, dependent on the size of the wound, are appropriate to effectively maintain wound  
24 closure and promote wound healing in approximately 5-7 days.

25 **16** Finally, clean the skin around the wound with sterile saline to remove any blood from surgical  
26 procedure.

27

## 28 **Post-Surgical Welfare**

29 **17** Dress the wound with Betadine spray to help prevent infection.

- 1 **18** Return rats to their home cage in recovery room with a few moistened and dry pellets placed in  
2 cage to aid feeding post-surgically, routinely housed in groups of 4. Monitor general behaviour  
3 and body weight of animals post-surgery to ensure no unexpected effects of surgery are  
4 observed. Post-surgical local analgesic agents are not routinely administered, unless required,  
5 as these may influence responses observed under test conditions. **! CAUTION** Typically we  
6 observe no additional effects of the surgery with autotomy observed extremely rarely (1 animal  
7 in over 300 surgeries). In our experience, body weight of the animals may be affected marginally  
8 over the first 48 hours post-surgery, but following this time anticipated increases in body weight  
9 are observed, any alteration in this may indicate a problem with the surgical procedure.
- 10 **19** Remove wound clips using an appropriate clip remover 7 days post-surgery. An important  
11 observation is that TNT rats display an abnormal posture of the ipsilateral hindpaw, placing  
12 weight through the medial surface of hindpaw rather than flat through the plantar surface,  
13 which does not appear to influence other behaviours. **! CAUTION** Care must be taken to ensure  
14 that the wound has appropriately healed in this time, for the welfare of the animal clips must  
15 not be taken out prior to sufficient wound healing, or left in longer than necessary which may  
16 potentially cause the animal additional pain. **! CAUTION** Wound clips have sharp metal points  
17 and must be handled with care with clip removers and disposed of in a sharps bin.

#### 18 **Outcome Parameter: Mechanical Hypersensitivity**

- 19 **20** Prior to testing habituate rats to testing equipment, initially three sessions are carried out on  
20 three separate days as described below. Commonly for testing of established pain this training  
21 should be carried out at approximately 14 days post-surgery, when we anticipate robust  
22 hypersensitivity to have developed in the majority of animals. However, this can be carried out  
23 prior to surgery or from 1 day post-surgery, allowing rats at least 24 hours to recover from  
24 surgery.
- 25 A. On the first day of habituation place rats in perspex arenas and leave to habituate  
26 for approximately 45 mins. **! CAUTION** Length of habituation time set to allow rats  
27 time to cease exploratory behaviour and settle in arenas, presence of experimenter  
28 in room during this period is not essential. Following habituation to the arena apply  
29 the 15 g von Frey filament to the lateral, plantar surface of contralateral (as  
30 illustrated in Figure 3), and then ipsilateral hindpaw for approximately 6 secs.  
31 Repeat this procedure using 8 g filament. These applications during the training  
32 period are to reduce any effect of novelty following mechanical stimulation, which  
33 could potentially mask true thresholds.

1 B. On the second day of habituation place rats into the perspex arena they were  
2 habituated to previously (numbering arenas and only cleaning bases between  
3 sessions appears to aid rats recognition of the arena and, thus, reduce time taken to  
4 habituate on subsequent sessions) and allow sufficient time for habituation. Repeat  
5 stimulation with von Frey filaments as previously described, except using 8 g then 4  
6 g filaments.

7 C. On the final day of training repeat step B, but apply a 4 g von Frey filament, then  
8 carry out a full assessment of mechanical hypersensitivity using the up-down  
9 method <sup>29</sup>. Briefly, a range of stimulations should be applied to each hindpaw in  
10 turn, starting with the 2 g filament, increasing or decreasing according to whether a  
11 reflex paw withdrawal is observed, to ascertain the threshold for response/no  
12 response. This method differs from other threshold measures as following threshold  
13 detection four further stimulations (exception when highest or lowest are  
14 surpassed) should then be applied to determine whether the specific threshold  
15 value lies closer to the lower filament in the threshold (highest no response value) or  
16 the higher filament (first filament which induces a response). This method produces  
17 a 50% paw withdrawal threshold (PWT) score calculated using a statistical formula  
18 used by Chaplan et al. In their publication:

$$19 \quad 50\% \text{ g threshold} = (10^{[Xf + k\delta]})/10,000 \quad 29$$

20 Where Xf represents the log value of the last filament tested and kδ is a constant  
21 found in a table of values. The only exceptions to this formula used are if the animal  
22 doesn't respond to any filament, as 15 g is the highest filament tested use 15 g as  
23 cut off for this method of testing. Similarly, if an animal responds to all filaments  
24 down to 0.4 g then use a cut off of 0.25 g.

25 Always test the contralateral hindpaw prior to the ipsilateral hindpaw. **! CRITICAL**  
26 **STEP** We have attempted to standardise our testing further, by testing contralateral  
27 followed by ipsilateral hindpaw of 3 rats, each filament of each rat in turn, before  
28 moving on to next 3 rats. We believe this is important to ensure consistency  
29 between experimenters and allow a relatively standard time between testing  
30 between applications helping to prevent against possible overstimulation. **!**  
31 **CRITICAL STEP** Due to the complex nature of testing a group of animals using this  
32 method, which may involve as many of 9 stimulations per hindpaw, we would  
33 recommend the use of a template (we regularly use an Excel, Microsoft Office

1 spreadsheet) to enter response (X), no response (O) and final filament log unit into,  
2 which then automatically calculates 50% PWT. This reduces opportunity for errors  
3 and allows a quick checking mechanism (pattern must be complete for a 50% PWT  
4 to be calculated) to ensure the appropriate stimulations have been carried out.

5 **21** Assess responsiveness to von Frey filaments using the up-down method described above on any  
6 given time point post-surgery to provide baseline data for each animal prior to testing with  
7 potential analgesic compounds<sup>33</sup>.

8 **22** Subsequently further calculation of responsiveness illustrated by 50% PWT value can then be  
9 obtained using the same process, for example as part of a time course post-surgery or at  
10 appropriate time points following drug treatment, and analysed using statistics relevant to the  
11 specific study design. Importantly, using 50% PWT scoring as opposed to threshold values  
12 produces lower values as these are average values. Literature evidence suggests that values  
13 above 8-10 g represent noxious stimulation, with the majority of studies using manually applied  
14 von Frey filaments to calculate PWT measures demonstrating that this force represents the  
15 threshold required to elicit a behavioural response in naive rats, or contralateral hindpaws  
16 <sup>19,29,34,35</sup>. This converts to a 50% PWT score of approximately 7-8 g, thus, we consider a 50% PWT  
17 score of lower than 3 g in ipsilateral hindpaw and greater than 8 g in the contralateral to  
18 represent unilateral hypersensitivity in a TNT rat.

19 **23** To test potential analgesic compounds carry out the study in a randomised and blind manner.

20 **▲ CRITICAL STEP** Assessment of hypersensitivity should always be carried out fully-blind and  
21 randomised to avoid bias in results. To ensure animals are randomised and displaying pain,  
22 baseline hypersensitivity measures are always taken at the start of each study. Animals are then  
23 randomised based on their baseline scores by a second experimenter and dosed with test  
24 compound using a wide range of dosing techniques, such as oral, sub-cutaneous and inter-  
25 peritoneal, which in our experience commonly do not significantly change 50% PWT score alone  
26 as assessed by effect in vehicle-treated animals (data not shown). Investigation of the effect of  
27 the test compound on 50% PWT score, with effect of compound compared to vehicle treatment  
28 at each time point tested, should then be made. **▲ CRITICAL STEP** Animals should always have  
29 a reasonable time to re-settle into perspex arenas following dosing prior to testing, and is  
30 important to consider method of dosing and the inclusion of appropriate vehicle controls. Also  
31 important to consider is the number of time points tested in a single day, in our hands, we find  
32 that responses become variable and inconsistent, possibly due to over stimulation, after 3 time  
33 points per study day.

1

## 2 **Timing**

3 Steps 1-8, pre-surgical preparation approximately 5 mins per animal

4 Steps 9-17, surgical procedure approximately 10 mins per animal

5 Steps 18-19, post-surgical welfare over first 48 hrs post-surgery

6 Step 20, clip removal, few seconds per clip

7 Step 21, measurement of mechanical hypersensitivity, requires 30 -45 mins habituation to arena  
8 then approx 5 – 10 mins per rat. This stage is very dependent on experimenter competency and  
9 confidence in up-down technique, ideally, assessment of 12 rats should take approximately 30-40  
10 mins.

11

## 12 **Anticipated Results**

13 Following the TNT surgery as outlined above, we typically observe a robust hypersensitivity  
14 compared to naive or contralateral values, from 14 days post-surgery through to the final time point  
15 tested (currently around 70 days). Hypersensitivity is present from day 1 post-surgery but in a lower  
16 proportion of animals compared to later time points post-surgery, and data can appear more  
17 variable<sup>33</sup>. This model allows potential re-use of animals for efficacy studies following compound  
18 washout periods, with 100% of rats routinely displaying hypersensitivity in the ipsilateral hindpaw,  
19 although responses may vary on a day-to-day basis, due to individual changes in behaviour.  
20 Typically 80% of rats display unilateral hypersensitivity on any one test day, as defined by 50% PWT <  
21 3 g on ipsilateral hindpaw and > 8 g on contralateral hindpaw. Naive and contralateral 50% PWT  
22 values are commonly around 8- 15 g 50%PWT, thus demonstrating a window of around 10-12 g  
23 between injured and un-injured hindpaw. Experimentally we compare the effect of the treatment  
24 groups with the vehicle-control at the test time point using ANOVA to assess efficacy of the  
25 compound. Baseline values are also compared between groups to ensure a consistent even starting  
26 point, but are not regularly used to compare with test time point as this does not take into account  
27 any effect of testing per se.

28

29 As previously mentioned we have demonstrated that several clinically relevant compounds reverse  
30 mechanical hypersensitivity in TNT rats demonstrating the potential of TNT as a predictive model, to  
31 this end we have confirmed the effectiveness of several analgesics used in the clinic. For us,



1 validation of the model is routinely carried out using pregabalin (10 or 20 mg/kg, PO, 1-3 hrs post-  
2 dose) or gabapentin (100 mg/kg, PO, 1-2 hrs post-dose), which significantly reverses mechanical  
3 hypersensitivity in the ipsilateral hindpaw compared to vehicle-treated rats, as demonstrated for  
4 gabapentin in Figure 5, along with typical baseline 50% PWT values observed from day 14 post-  
5 surgery. These doses and testing time points were chosen based on pharmacokinetic data  
6 confirming sufficient plasma exposure at this time to produce efficacy (from previous studies, data  
7 not shown). Clearly, determining exposure of compounds and having some knowledge of the  
8 pharmacokinetic profile is useful to fully interpret this type of study. Providing this comprehensive  
9 protocol with surgery details and training/testing procedures for mechanical hypersensitivity is to  
10 provide sufficient information to allow comparison with previously published data using this model,  
11 or other commonly used ligation models, to consider possible reasons for similarities and differences  
12 observed in behaviour and drug responsiveness.

13

1 **Author Contributions**

2 D.R. contributed to establishment of protocol, generation of data and wrote the manuscript. H.W.,  
3 J.R.N. and I.M. contributed to design of experiments and writing of the manuscript. L.C. and G.B.  
4 contributed to the design of experiments, (specifically L.C. contributing to set up of fully-blind  
5 protocol and Dixon up-down method). H.W. and H.G. carried out surgical procedures and  
6 contributed to writing of the manuscript.

7

8 **Acknowledgements**

9 We would like to thank members of the Comparative Medicine and Pain *in vivo* behaviour teams at  
10 Pfizer, Sandwich, past and present, for their contribution to the development of this model.

11

12 **Competing Interests Statement**

13 The authors declare that they have no competing financial interests.

14

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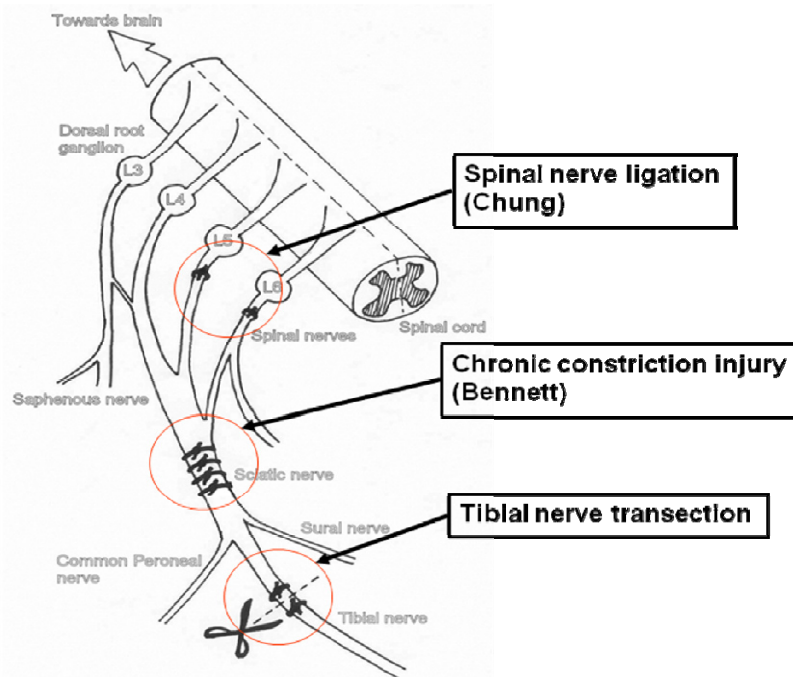
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1 **Figures**

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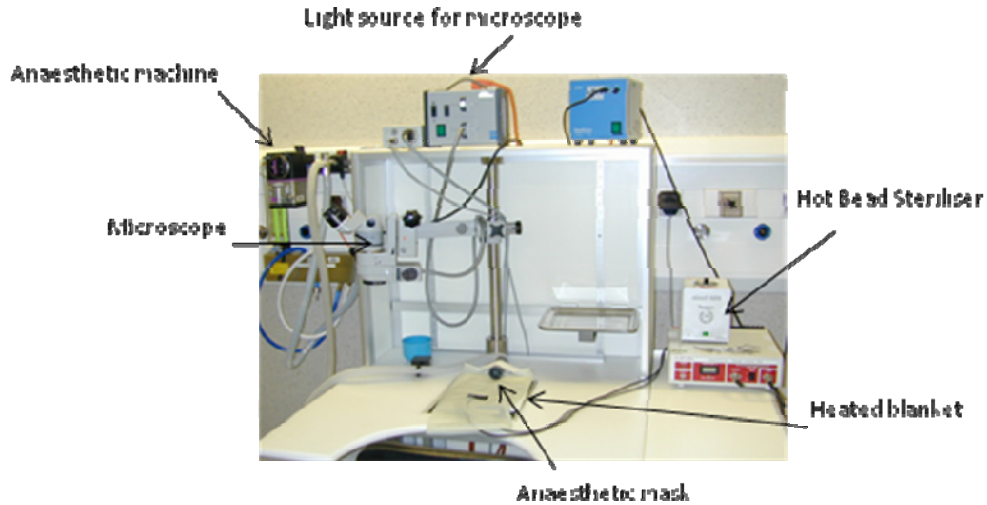
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4 **Figure 1.**

5 Illustration of anatomical location and nature of the tibial nerve transection (TNT) injury compared  
6 with two other commonly used peripheral nerve injury models. Demonstrated by dark suture and  
7 tie around the nerves are ligations, with spinal nerve ligation (SNL) model consisting of tight ligation  
8 of L5 and L6 branches of the spinal nerve distal to the dorsal root ganglia. The chronic constriction  
9 injury (CCI) model involves tying four loose ligatures around the sciatic nerve, formed from the spinal  
10 nerves, prior to the trifurcation into tibial, common peroneal and sural nerves. TNT injury differs  
11 from these models with the injury induced by transecting the tibial branch of the sciatic nerve  
12 between two ligatures which are placed approximately 2mm apart. Another commonly used model,  
13 the spared nerve injury (SNI) model is similar to the TNT model, but with transection of both tibial  
14 and common peroneal nerves. Redrawn and adapted from <sup>18</sup>.

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2 *Figure 2.*

3 An example of the equipment set-up used to perform tibial nerve transection (TNT) surgery. The rat  
4 is placed on the homoeothermic heated blanket following induction of anesthesia, with its head  
5 facing away from the surgeon. Surgical anaesthesia is maintained using a nose cone to supply  
6 Isoflurane anaesthetic in oxygen. The surgery is carried out on this set-up for optimal speed of  
7 surgery and ease of animal monitoring, prior to return of the rat to its home cage for recovery.

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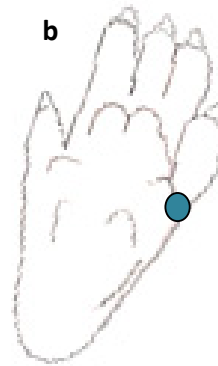
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3 **Figure 3.**

4 Application of a von Frey filament to the plantar surface of a rat hindpaw is used to measure  
5 mechanical allodynia. (a) In an individual perspex arena, mounted on rows of metal bar flooring, the  
6 lateral plantar aspect of the rat hindpaw can be accessed. Following the application of von Frey  
7 filaments in ascending or descending force dependent on the response a threshold response can be  
8 obtained. The filament is applied to the lateral aspect of the hindpaw in the approximate area show  
9 in (b) highlighted by a blue circle. 50% paw withdrawal threshold (PWT) scores are obtained by  
10 continuing in the up-down pattern for 4 subsequent readings after the initial threshold and inputting  
11 the data into a statistical formula as described <sup>25</sup>.

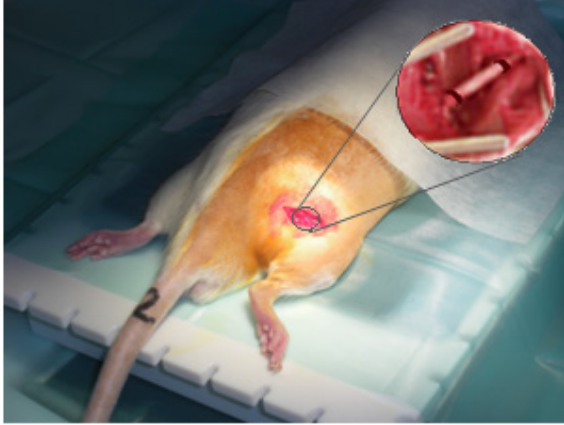
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2 *Figure 4.*

3 The tibial nerve branch of the sciatic nerve is the final, and thickest, nerve portion to appear from  
4 the sciatic nerve. Ligation, as illustrated in this surgical photograph, and transection between these  
5 ligations occurs on this branch before the subsequent trifurcation of the tibial nerve.

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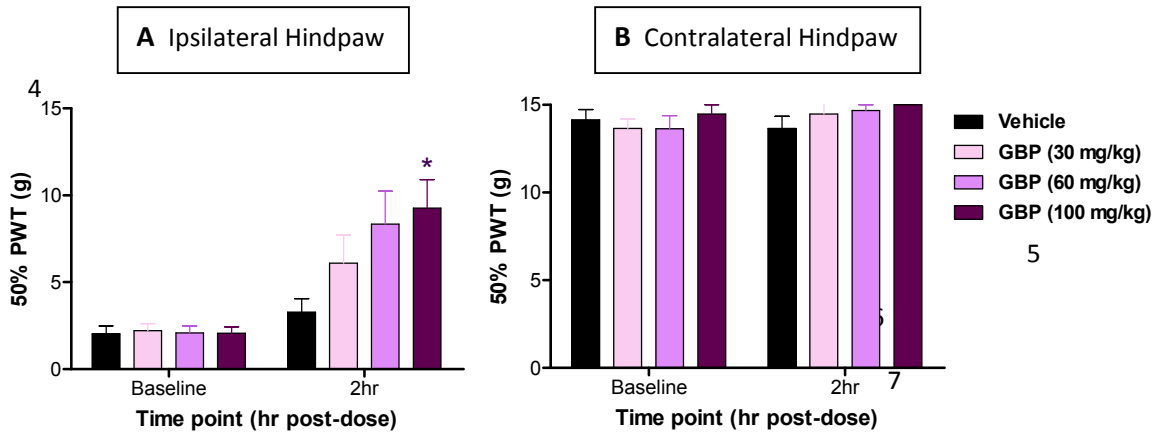
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**Figure 5.**

Effects of the clinically used compound gabapentin, on TNT-induced mechanical allodynia. Following TNT surgery rats displaying 50% paw withdrawal threshold (PWT) baseline values of lower than 3 g in the ipsilateral hindpaw and > 8 g in the contralateral hindpaw are selected for study (approximately 80 % of rats tested at a specific time point). This study represents anticipated values when tested from 14 days post-surgery through to approximately 70 days post-surgery, with this particular study carried out at approximately 50 days post-TNT surgery. Rats were then randomised according to baseline values, to ensure even spread of scores across treatment groups, and dosed by a second experimenter, thus maintaining blinding of experimenter assessing behaviour. Gabapentin at 100 mg/kg significantly attenuated the level of mechanical allodynia observed in TNT rats when compared to vehicle-treated rats, in a dose-related manner. Data are expressed as mean + SEM (n = 9 rats per group), ANOVA used; \* P < 0.05 vs vehicle.

