Journal of Visualized Experiments

Modified Spared Nerve Injury (SNI) Surgery Model of Neuropathic Pain in Mice

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63362R2
Full Title:	Modified Spared Nerve Injury (SNI) Surgery Model of Neuropathic Pain in Mice
Corresponding Author:	Zhonghui Guan University of California San Francisco San Francisco, CA UNITED STATES
Corresponding Author's Institution:	University of California San Francisco
Corresponding Author E-Mail:	Zhonghui.Guan@ucsf.edu
Order of Authors:	Liangliang He
	Wenxing Zhao
	Maalveka Ilango
	Na Zhao
	Liqiang Yang
	Zhonghui Guan
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Neuroscience
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	San Francisco, CA, USA
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please confirm that you have read and agree to the terms and conditions of the video release that applies below:	I agree to the Video Release
Please provide any comments to the journal here.	

TITLE:

Modified Spared Nerve Injury Surgery Model of Neuropathic Pain in Mice

AUTHORS AND AFFILIATIONS:

Liangliang He[1,2,*], Wenxing Zhao[1,*], Maalveka Ilango[2,3], Na Zhao[2], Liqiang Yang[1], Zhonghui Guan[2]

[1]Department of Pain Management, Xuanwu Hospital, Capital Medical University, No. 45 Changchun Street, Xicheng District, Beijing, China

[2]Department of Anesthesia and Perioperative Care, University of California San Francisco, San Francisco, California, USA

[3]University of Washington, Seattle Campus, 1400 NE Campus Parkway, Seattle, Washington, USA

[*]These authors contributed equally.

Email addresses of co-authors:

Liangliang He	(liangliang.he@ucsf.edu)
Wenxing Zhao	(zhaowxing@yeah.net)
Maalveka Ilango	(ilanmaalve1@gmail.com)
Na Zhao	(75475608@qq.com)
Liqiang Yang	(yangliqiangxwpain@outlook.com)
Zhonghui Guan	(zhonghui.guan@ucsf.edu)

Corresponding author:

Liqiang Yang	(yangliqiangxwpain@outlook.com)
Zhonghui Guan	(zhonghui.guan@ucsf.edu)

SUMMARY:

The modified surgery is a simplified method for mouse or rat spared nerve injury model that requires only one ligation and one cut to injure both common peroneal and sural nerves.

ABSTRACT:

Spared nerve injury (SNI) is an animal model that mimics the cardinal symptoms of peripheral nerve injury for studying the molecular and cellular mechanism of neuropathic pain in mice and rats. Currently, there are two types of SNI model, one to cut and ligate the common peroneal and the tibial nerves with intact sural nerve, which is defined as SNIs in this study, and another to cut and ligate the common peroneal and the sural nerves with intact tibial nerve, which is defined as SNIt in this study. Because the sural nerve is purely sensory whereas the tibial nerve contains both motor and sensory fibers, the SNIt model has much less motor deficit than the SNIs model. In the traditional SNIt mouse model, the common peroneal and the sural nerves are cut and ligated separately. Here a modified SNIt surgery method is described to damage both common peroneal and sural nerves with only one ligation and one cut with a shorter procedure time, which is easier to perform and reduces the potential risk of stretching the sciatic or tibial nerves, and produces similar mechanical hypersensitivity as the traditional SNIt model.

INTRODUCTION:

Nerve injury-induced neuropathic pain following surgery or trauma has a significant economic burden that impairs quality of life. A host of nerve injury models, including spinal nerve ligation (SNL)[1], chronic constriction injury (CCI)[2] to the sciatic nerve, partial sciatic nerve ligation (pSNL)[3], sciatic nerve transaction (SNT)[4] and spared nerve injury (SNI)[5,67,8], were successfully developed to mimic the cardinal symptoms of peripheral nerve injury in rats and mice for studying the molecular and cellular mechanism of neuropathic pain[6–10]. However, each surgical model has its benefits and limitations, therefore particular attention should be given to exploring and developing the surgery models[10].

The rodent SNI model produces long-term hypersensitivity to mechanical stimulation. However, it is somewhat confusing because there are two different SNI models. The initial SNI model was developed in Woolf's lab, in which the common peroneal and the tibial nerves were injured, leaving the sural nerve intact[5,6]. The second SNI model was developed in Basbaum's lab, in which the common peroneal and the sural nerves were injured, leaving the tibial nerve intact[7,8]. The initial Woolf's model is defined as SNIs here because the sural nerve is left intact, and Basbaum's model is defined as SNIt here because the tibial nerve is left intact. Because the sural nerve is purely sensory whereas the tibial nerve contains both motor and sensory fibers, the SNIt model has much less motor deficit than the SNIs model. However, unlike the SNIs model, mice in the SNIt model do not develop thermal hypersensitivity, but mechanical hypersensitivity develops in both models. Although the SNIt model is a relatively easy procedure, it requires the ligation of the sural and common peroneal nerves separately with the potential risk of stretching the sciatic or tibial nerves[6–9].

The common peroneal, tibial, and sural nerves are three branches of the sciatic nerve and can be clearly identified at the superior edge of the gastrocnemius muscle (**Figure 1**): the tibial nerve goes under the gastrocnemius muscle, and the common peroneal (cephalad side) and sural nerve (caudal side) are above the gastrocnemius muscle[11]. Based on its anatomical features, a modified mouse SNIt surgery procedure was developed to ligate the common peroneal and sural nerves together with only one nerve-ligation and one nerve-cutting, which results in shortened procedure-time.

PROTOCOL:

Animal experiments were approved by UCSF Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory animals. Adult C57BL/6 mice weighing 20–30 g was used in this study. The von Frey assessment was performed between 1:00 pm and 3:00 pm.

1. Anesthesia and mouse preparation

1.1 Place a mouse in a plastic chamber filled with 2% isoflurane in O_2 at a flow rate of 1.0 L/min until it is fully anesthetized.

1.2 Cover the eyes with ophthalmic ointment using a cotton-wool applicator.

1.3 Place the snout of the mouse into a flexible nose cone with 2% isoflurane flow throughout the surgical procedure.

1.4 Place the mouse in a right lateral position. Keep the left leg up with knee flexion and secure it with adhesive tape.

1.5 Remove the hair around the thigh and knee area with an electric shaver and disinfect the skin with 70% alcohol.

1.6 Ensure sufficient anesthesia depth before surgery by testing no response to pinch stimulus on the hind limb or tail with tweezers as the standard.

NOTE: No local anesthetic or NSAID was used before and after performing the SNIt model because local anesthetic and NSAID significantly reduce the neuropathic pain behavior after SNIt.

2. Modified SNIt surgery

2.1 Cut a 1 cm incision starting at the first 1/3 of the horizontal line crossing the knee with an approximately 30° angle from the vertical line with scissors (**Figure 2A**).

2.2 Two white lines can be visualized under the biceps femoris muscle (BFM) after separating skin incision, with the medial (cephalad) thick line as the femur and lateral (caudal) thin line as sciatic nerve (**Figure 2B**).

2.3 Blunt dissect BFM along the caudal white line with curved micro forceps and micro scissors to expose the sciatic nerve. Avoid blood vessel damage during blunt dissection. If accidental vessel damage occurs, use sterile cotton-wool swabs to absorb blood and apply proper pressure to stop bleeding.

2.4 Differentiate the three branches from the sciatic nerve at the superior edge of the gastrocnemius muscle. The tibial nerve is the one with the biggest diameter passing under the gastrocnemius muscle, whereas the sural nerves (lateral, the smallest diameter) and the common peroneal nerve (medial) run above the gastrocnemius muscle (**Figure 2C** and **Figure 3A**).

2.5 Depending on how BFM is dissected and opened, visualize the common peroneal nerve as lateral (**Figure 2C**) or medial (**Figure 3A**) to the tibial nerve.

2.6 Separate the common peroneal and the sural nerves from the neighboring tissues using curve micro forceps.

2.7 Ligate the common peroneal and sural nerves together with a 6-0 suture, as both nerves run above the gastrocnemius muscle, but the tibial nerve passes under the gastrocnemius muscle

(**Figure 2D** and **Figure 3B,C**). Observe the limb for contraction following the tight ligature. For the traditional method, ligate the common peroneal and sural nerves separately.

2.8 Cut nerve at a distal part within 2–4 mm of the ligation with a pair of micro scissors. Ensure that the tibial nerve remains untouched during the whole procedure. For the traditional method, cut the common peroneal and sural nerves at the distal part within 2–4 mm of the ligation and remove a 2 mm section, separately.

2.9 Close the muscular layer with a 6-0 silk suture and the skin incision with wound clips.

2.10 After surgery, return the mice to the animal room until full recovery from anesthesia. Check daily for intact incisions, normal food intake, water consumption, general body condition, regular movements, and grooming. Remove wound clips 7–14 days after surgery.

3. von Frey assessment for mechanical threshold

3.1 Acclimatize the mice for 6 days to the testing room environment and testing materials to perform the von-Frey assessment. Place the mice in clear plastic cylinders on an elevated wire mesh grid for 1 h of habituation. Place white papers between each cylinder to prevent any visual cue from each testing animal.

3.2 During this period, perform habituation every 2 days, and measure the baseline of von Frey monofilaments under the mid-plantar of the hind paw after the last habituation.

3.3 Perform von Frey assessment with a blind tester. Stimulate the mid-plantar of the hind paw with von Frey filaments by using the up-down paradigm[12]. Apply the von Frey filament to the plantar surface perpendicularly with applied force to cause slight curling.

3.4 Verify positive responses as sudden paw withdrawal, sudden flinching, or sudden paw licking. Exert the next stimulus at an interval of 5 s to avoid the influence of the previous stimulus.

3.5 Confirm mechanical threshold per paw by taking an average of 3 sessions.

3.6 Based on the 50% hind paw withdrawal threshold decided by the up-down method, use the percent response method with 0.16 g filament to assess the difference further.

3.7 Record the percentage of the positive responses after 10 stimuli of 0.16 g filament applied to the mid-plantar of the hind paw regardless of the responses.

3.8 Perform the von Frey assessments on pre-surgery day 1, and post-surgery on days 1, 3, 5, 7, and 14.

4. Statistical Analysis

4.1 Report normally distributed continuous data as mean ± standard error of the mean (SEM). Analyze the continuous data with 2-tailed t-test or two-way repeated-measures ANOVA. Process all data using statistical analysis software, with statistical significance at the level of $p < 0.05$.

REPRESENTATIVE RESULTS:

The comparison of procedure time between modified and traditional methods.

The procedure time from the beginning of cutting the skin to the end of closing skin was recorded in 5 mice with the modified approach and 5 mice with the traditional approach, respectively. A minimal number of animals was used to obtain results with statistical significance. Compared with the control of the traditional approach[7,13] to perform SNIt, the modified approach took almost half the amount of procedure time (236.2 ± 28.6 s *vs.* 422.6 ± 53.8 s, p=0.0156; **Figure 4**).

The comparison of mechanical hypersensitivity between the two methods.

No difference of von Frey assessment (manual) was observed in the two groups at baseline (1.05 ± 0.10 g *vs.* 0.96 ± 0.13 g, p=0.9405). Compared with the traditional method (n=9), the modified method (n=14) induced similar mechanical hypersensitivity on the ipsilateral side from post-operative day 1 to post-operative day 14 (0.33 ± 0.09 g *vs*. 0.32 ± 0.05 g, p>0.9999, at post-operative day 1; 0.19 ± 0.02 g *vs.* 0.27 ± 0.06 g, p=0.9485 , at post-operative day 3; 0.20 ± 0.06 g *vs*. 0.15 ± 0.05 g, p=0.9979, at post-operative day 5; 0.13 ± 0.02 g *vs.* 0.15 ± 0.03 g, p>0.9999, at post-operative day 7; and 0.14 ± 0.02 g vs. 0.19 ± 0.03 g, p=0.9937, at post-operative day 14; **Figure 5A**).

Compared with percentage response between traditional (n=5) and modified method (n=5), similar mechanical hypersensitivity was observed on the ipsilateral side from baseline (4.00% ± 2.45% *vs.* 6.00% ± 2.45%, p>0.9999) to post-operative day 1 (20.00% ± 3.16% *vs.* 12.00% ± 3.74%, p=0.8987), day 3 (30.00% ± 5.48% *vs.* 32.00% ± 4.90%, p>0.9999), day 5 (36.00% ± 9.27% *vs.* 30.00% ± 5.48%, p=0.9730), day 7 (32.00% ± 8.00% *vs.* 36.00% ± 4.00%, p=0.9968), and day 14 (38.00% ± 5.83% *vs.* 36.00% ± 7.48%, p>0.9999; **Figure 5B**).

No neural reconnection between the distal and remaining stump at post-operative day 14.

Because the axon regeneration proceeds at a rate of 1–3 mm/d[14], whether there is nerve regeneration at post-operative day 14 should be taken into account. On the post-operative day 14, the modified SNI mice were perfused intracardially with 0.1 mol/L phosphate-buffered saline followed by a phosphate-buffered 10% formalin fixative, and the nerve ligation site was dissected and exposed. No neural reconnection was observed between the distal and remaining stumps (**Figure 3D**).

FIGURE AND TABLE LEGENDS:

Figure 1. Illustration of the 3 branches of the sciatic nerve that are separated at the superior edge of the gastrocnemius muscle (GM) around the knee area. The tibial nerve runs under the GM muscle, and the common peroneal nerve and sural nerve run above the GM muscle. 1: sciatic nerve; 2: tibial nerve; 3: common peroneal nerve; 4: sural nerve. Abbreviations: BFM = biceps femoris muscle; GM = gastrocnemius muscle.

Figure 2. Illustration of surgical procedure in modified SNI mice. (**A**) In a lateral position, the left leg is upside with knee flexion. The yellow horizontal line (1) indicates the horizontal line crossing the knee level, and the yellow dashed arrow (2) indicates the incision. (**B**) Following a skin incision, two white lines can be visualized under the biceps femoris muscle (BFM). The cephalad one (3) is the femur, and the caudal one (4) is the sciatic nerve. (**C**) After careful dissection of BFM, the sciatic nerve (5) and its three branches can be visualized: the tibial (6), common peroneal (7), and sural (8) nerves. (**D**) The common peroneal (7) and sural (8) nerves were ligated together. 1: horizontal line crossing knee; 2: cut incision; 3: femur on the underneath; 4: sciatic nerve on the underneath; 5: Sciatic nerve; 6: tibial nerve; 7: common peroneal nerve; 8: sural nerve; 9: white curve line indicates knee.

Figure 3. Illustration of sciatic, tibial, sural, and common peroneal nerves with the ligation of modified SNI in intracardially perfused mice. (**A**) Sciatic nerve (1) and its three branches: the tibial nerve (2) passing under the gastrocnemius muscle (5), and the common peroneal (3) and the sural (4) nerves running above the gastrocnemius muscle. (**B**) Suture needle crossing under common peroneal and sural nerves together. (**C**) A 6-0 nylon suture was used for the ligation of common peroneal and sural nerves together. (**D**) No reconnection between distal and remaining nerve stump at post-operative day 14 was observed. 1: Sciatic nerve; 2: common peroneal nerve 3: tibial nerve; 4: sural nerve; 5: gastrocnemius muscle; 6: ligation.

Figure 4. The modified method is faster than the traditional SNI method to perform. Compared to the traditional SNI method (n=5), the modified method (n=5) requires much less procedure time to perform. Analyses were performed using an unpaired t-test, and data are presented as mean ± SEM.

Figure 5. von Frey assessment of mechanical responsiveness in traditional and modified SNI models. (**A**) The modified SNI (n=9) and traditional SNI (n=14) models induced similar mechanical hypersensitivity on the ipsilateral side in 14-day follow-up. (**B**) The modified SNI (n=5) and traditional SNI (n=5) models acquired similar percentage responses on the ipsilateral side in a 14-day follow-up. Analyses were performed using two-way ANOVA with Sidak's multiple comparisons test. Data are presented as mean ± SEM.

DISCUSSION:

Compared to the traditional mouse SNIt method that ligates the common peroneal nerve and the sural nerve separately[6–9], the modified SNIt model has three advantages: (1) it has less risk of contracting or stretching sciatic or tibial nerves; (2) there is no need to remove the distal nerve stumps after nerve-cutting because by ligating the common peroneal nerve and the sural nerve together, the distal nerve stumps are anatomically separated from the proximal stumps. Indeed, anatomy dissection demonstrated that no nerve regeneration was observed 14 days after modified SNIt; (3) the procedure is much easier to perform with a much shorter procedure time.

It is critical to make skin incisions low around knee level, rather than high in the thigh near the hip, when performing the modified mouse SNIt. This is because the common peroneal, tibial, and

sural nerves have not branched out from the sciatic nerve at the high thigh area, which makes it challenging to separate the three branches. In contrast, at the superior edge of the gastrocnemius muscle near the knee area, the three branches from the sciatic nerve traverse separately and are easily visualized and identified, as the tibial nerve passing under the gastrocnemius muscle, and the common peroneal nerve on the cranial side and sural nerve on the caudal side running above the gastrocnemius muscle[11]. Interestingly, although the common peroneal nerve is medial to the tibial nerve at the thigh area, it usually crosses the tibial nerve to get closer to the sural nerve at the superior edge of the gastrocnemius muscle at the knee area in the lateral position with knee flexion, which makes it easier for ligating common peroneal and sural nerves together. Importantly, the tibial nerve remains intact without contacting or stretching when the common peroneal and sural nerves are ligated above the gastrocnemius muscle. In addition, when dissecting BFM to expose underneath nerves, it is important to avoid damaging blood vessels (lateral proximal genicular artery, popliteal artery, distal caudal femoral artery, etc.) located above the gastrocnemius muscle[15].

Although this modified SNIt model successfully develops neuropathic pain, some limitations are needed to be admitted. Because of damage to the common peroneal nerve, this could produce motor dysfunction with markedly extended hind leg[16]. Another, the mechanical hypersensitive area is innervated by intact nerve rather than injured nerve, but the neuropathic pain is often caused by the lesion or disease of the peripheral or central nervous system in clinical pain management[17]. Therefore, further study needs to be explored in the development of the neuropathic pain model.

In summary, this modified mouse SNIt method is a simplified SNI procedure with only one nerve-ligation and one nerve-cutting without any removal of nerve stumps. It is much easier to perform in producing nerve injury-induced mechanical hypersensitive, with reduced risk of sciatic or tibial nerves damage.

ACKNOWLEDGEMENTS:
Z.G. is supported by NINDS R01NS100801.

DISCLOSURES:
The authors declare no competing interests.

REFERENCES:
1. Chung, J. M., Kim, H. K., Chung, K. Segmental spinal nerve ligation model of neuropathic pain. *Methods in Molecular Medicine.* **99**, 35–45 (2004).
2. Vissers, K., Adriaensen, H., De Coster, R., De Deyne, C., Meert, T. F. A chronic-constriction injury of the sciatic nerve reduces bilaterally the responsiveness to formalin in rats: a behavioral and hormonal evaluation. *Anesthesia and Analgesia.* **97** (2), 520–525 (2003).
3. Malmberg, A. B., Basbaum, A. I. Partial sciatic nerve injury in the mouse as a model of neuropathic pain: behavioral and neuroanatomical correlates. *Pain.* **76** (1–2), 215–222 (1998).

4. Moore, K. A. et al. Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. *The Journal of Neuroscience: the official journal of the Society for Neuroscience.* **22** (15), 6724–6731 (2002).
5. Decosterd, I., Woolf, C. J. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain.* **87** (2), 149–158 (2000).
6. Cichon, J., Sun, L., Yang, G. Spared Nerve Injury Model of Neuropathic Pain in Mice. *Bio-Protocol.* **8** (6), e2777 (2018).
7. Shields, S. D., Eckert, W. A. 3rd, Basbaum, A. I. Spared nerve injury model of neuropathic pain in the mouse: a behavioral and anatomic analysis. *The Journal of Pain.* **4** (8), 465–470 (2003).
8. Guan, Z. et al. Injured sensory neuron-derived CSF1 induces microglial proliferation and DAP12-dependent pain. *Nature Neuroscience.* **19** (1), 94–101 (2016).
9. Richner, M., Bjerrum, O. J., Nykjaer, A., Vaegter, C. B. The spared nerve injury (SNI) model of induced mechanical allodynia in mice. *Journal of Visualized Experiments: JoVE.* (54), 3092 (2011).
10. Challa, S. R. Surgical animal models of neuropathic pain: Pros and Cons. *The International Journal of Neuroscience.* **125** (3), 170–174 (2015).
11. Kosaka, Y. et al. Development and persistence of neuropathic pain through microglial activation and KCC2 decreasing after mouse tibial nerve injury. *Brain Research.* **1733,** 146718 (2020).
12. Chaplan, S. R., Bach, F. W., Pogrel, J. W., Chung, J. M., Yaksh, T. L. Quantitative assessment of tactile allodynia in the rat paw. *Journal of Neuroscience Methods.* **53** (1), 55–63 (1994).
13. Bourquin, A. F. et al. Assessment and analysis of mechanical allodynia-like behavior induced by spared nerve injury (SNI) in the mouse. *Pain.* **122** (1–2), 14 e1–14 (2006).
14. Sulaiman, W., Gordon, T. Neurobiology of peripheral nerve injury, regeneration, and functional recovery: from bench top research to bedside application. *The Ochsner Journal.* **13** (1), 100–108 (2013).
15. Kochi, T. et al. Characterization of the arterial anatomy of the murine hindlimb: functional role in the design and understanding of ischemia models. *PLoS One.* **8** (12), e84047 (2013).
16. Omori, Y. et al. A mouse model of sural nerve injury-induced neuropathy: gabapentin inhibits pain-related behaviors and the hyperactivity of wide-dynamic range neurons in the dorsal horn. *Journal of Pharmacological Sciences.* **109** (4), 532–539 (2009).
17. Colloca, L. et al. Neuropathic pain. *Nature Reviews. Disease Primers.* **3**, 17002 (2017).

Figure 1

Figure 2

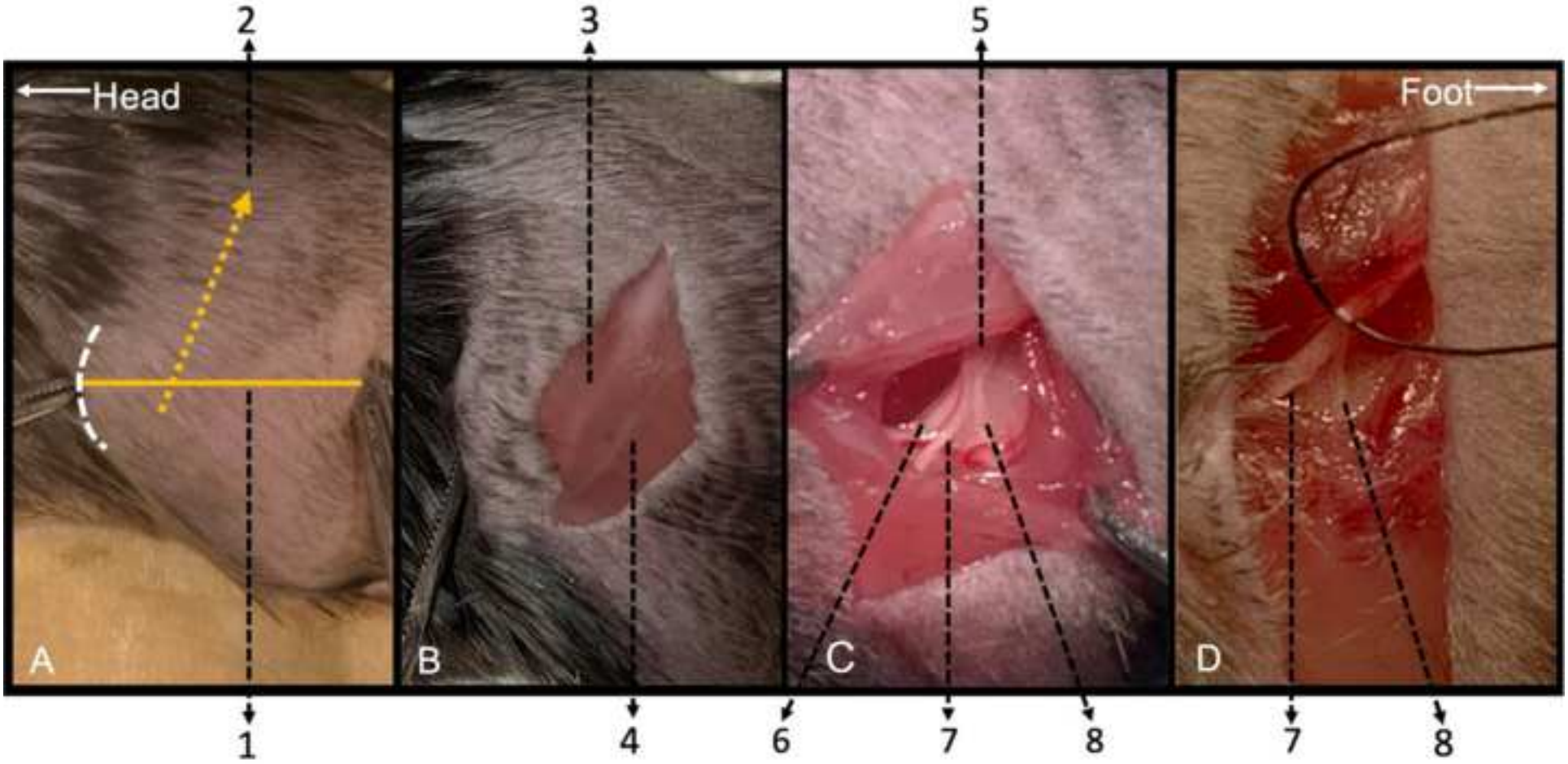


Figure 3

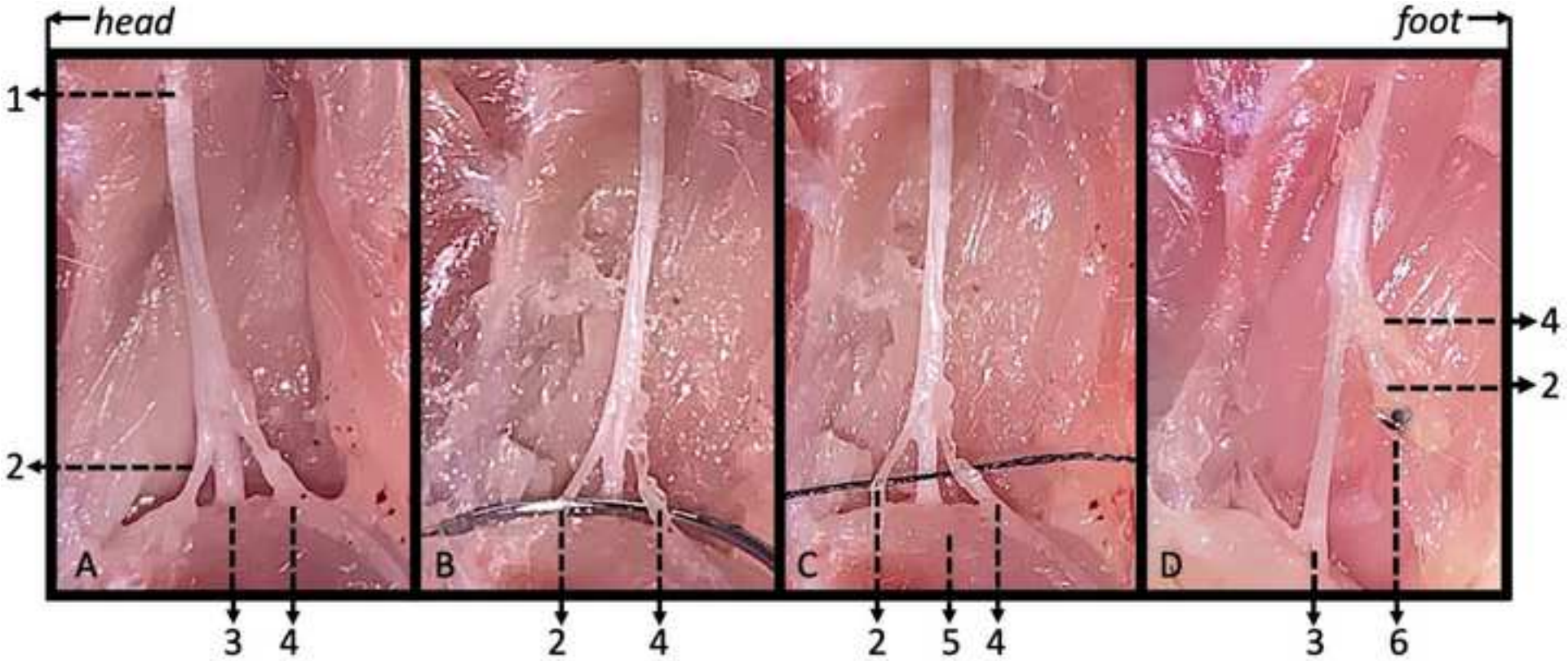


Figure 4

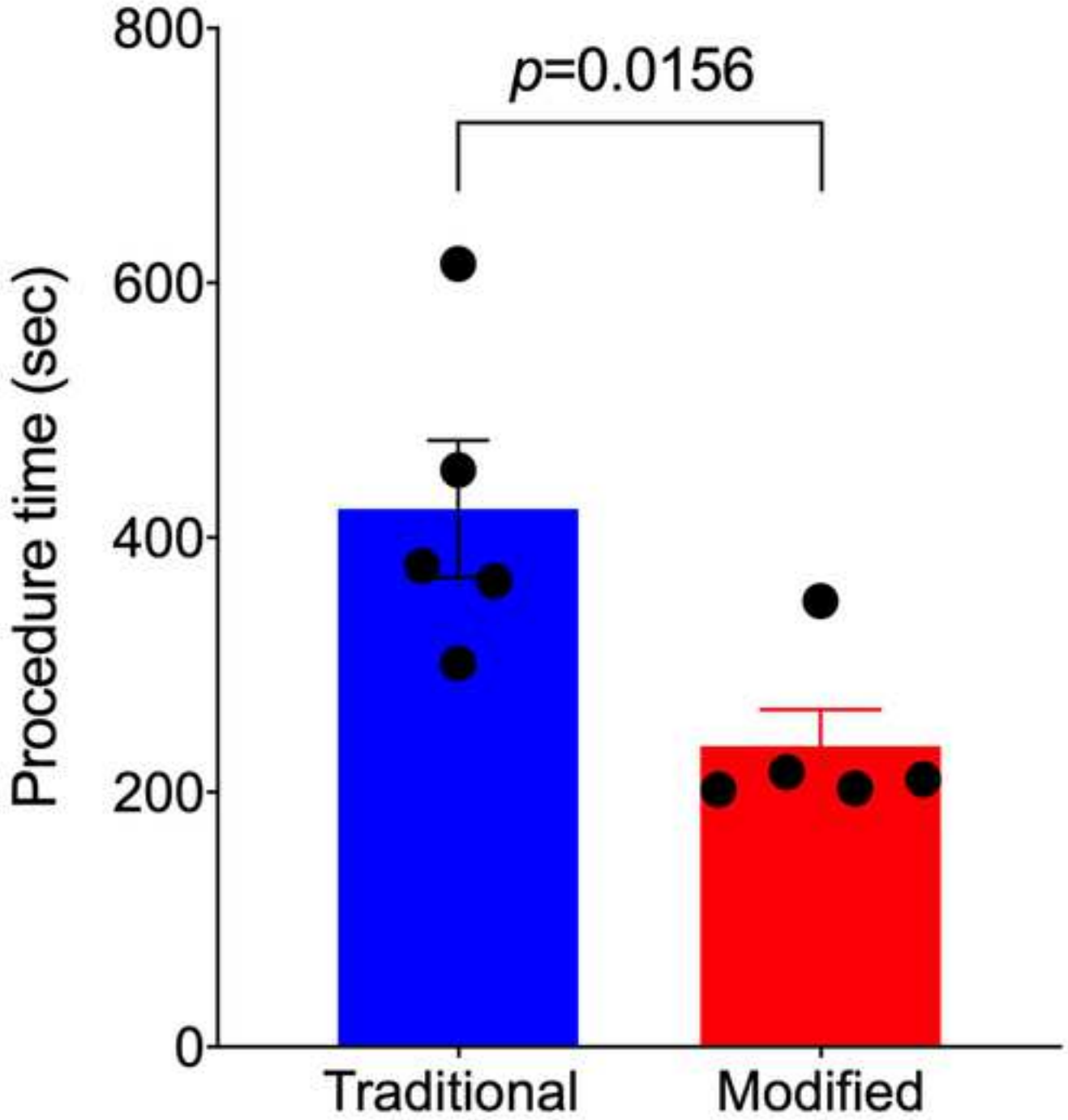
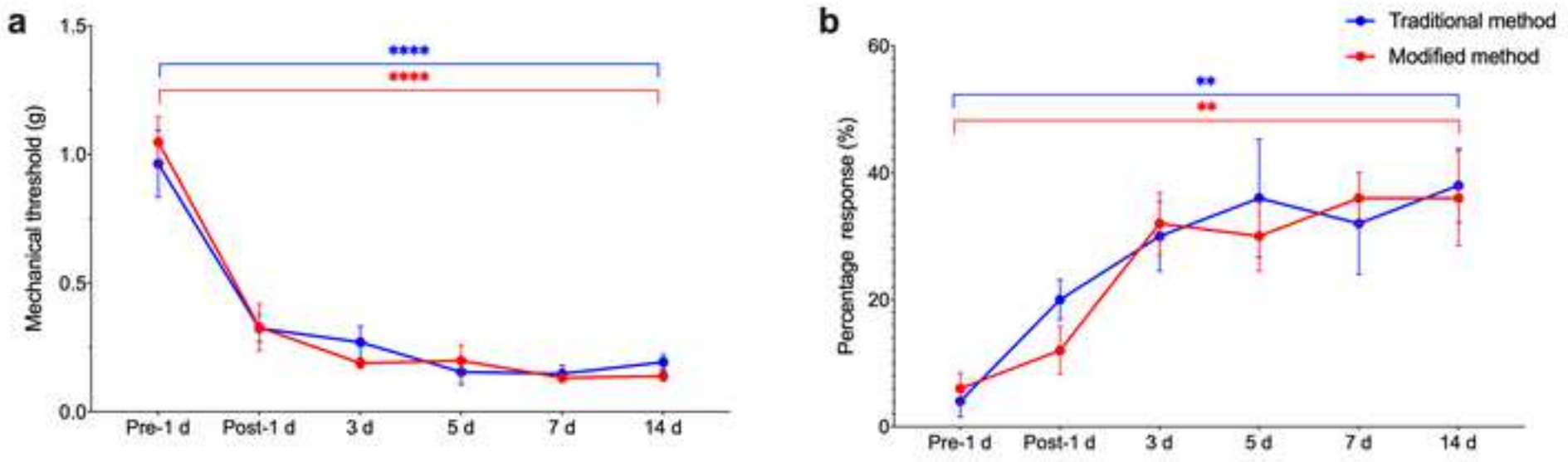


Figure 5



Reviewer #1:
Manuscript Summary:
The manuscript under consideration by He et al entitled "Simplified Spared Nerve Injury (SNI) Surgery Model of Neuropathic Pain in Mice" describes a fundamental tool that is essential to the pain/neuroscience research field. The manuscript is well written, and figures are of high quality with anatomical landmarks clearly demostrated. The procedure as decribed, would be highly reproducible. Many previsous publications describing this procedure did not clearly demonstrate anatomical landmarks. This method paper will be a great reference for the field to have.

Major Concerns:
No

Minor Concerns:
No

Response: Thanks for your kind comments.

Reviewer #2:
Manuscript Summary:
The present manuscript presents a simplified spared nerve injury surgery model, leading to the development of neuropathic pain in mice. The authors briefly point out the benefits of their methods compared to the surgery method established by e.g. Shields et al., 2003 (doi: 10.1067/S1526-5900(03)00781-8). The Von Frey test was used to assess the mechanical hypersensitivity, observed for a period of 14 days after spared nerve injury.

The abstract of the manuscript does not to sufficiently reflect the surgery procedure described in the following and, concerning the used animals, the figures appear to be contradictory to the written protocol. In general, the in vivo experiment should be reported in more detail, concidering the ARRIVE guidelines. Unlike the manuscript indicates, the authors did not develop the method. Both surgical methods to introduce a spared nerve injury (lesion of common peroneal nerve and sural or tibial nerve) as well as the application of one ligature and one section (detailed comments provided with according literature can be found below) have already been published. This needs clarification. Furthermore, the authors were not able to sufficently demonstrate the efficiency of the protocol, reflected by the stability of the introduced neuropathic pain, sufficiently. Only the mechanical allodynia was assessed by applying the Von Frey test. Mechanical hyperalgesia, cold allodynia, heat hyperalgesia and other aspects of neuropathic pain, e.g. spontaneous pain, reflecting neuropathic pain in humans, were not observed. Taking all points of criticism into consideration, I do not consider the manuscript for publication.

Major Concerns:
#1 "The traditional SNI mouse model requires ligating and cutting the common peroneal and the sural nerves separately, leaving the tibial nerve intact." (Line 26-28)
The cited publications (5-9) mainly induce a lesion of the common peroneal and the tibial nerve. Bourquin et al., 2006 (9) were even able to show that the simultaneous lesion of the common peroneal and the sural nerve does not lead to a mechanical hypersensitivity.
Furthermore, the authors might want to cite Decosterd and Woolf, 2000 (DOI:10.1016/S0304-3959(00)00276-1) who established the spared nerve injury as an animal model of persistent peripheral neuropathic pain.

It is confusing in SNI neuropathic pain model because there are two types of SNI models. The first one was initially developed by Decosterd and Woolf, 2000 in rat (added as citation 5), in which common peroneal and tibial nerves are injured. This method, which we defined as "SNIs" in the revised manuscript because the sural nerve remains intact, was eventually applied in mouse (citation 6). The second one was initially developed in Dr. Basbaum's lab in mouse and is normally used in our lab, in which peroneal and the sural nerves are injured (citation 7&8). In the revised manuscript we defined the second method as "SNIt" because the tibial nerve remains intact. Mouse sciatic nerve has three major branches, the tibial nerve, the common peroneal nerve, and the sural nerve. Both tibial and common peroneal nerves contain both sensory and motor fibers, and the sural nerve is a pure sensory nerve. As the result, the SNIt model developed by Dr. Basbaum's lab is superior that the SNIs model developed in Woolf's lab because the SNIt model has much less motor deficit. Our current manuscript is about the modification of SNIt model.

#2 "Although the mouse SNI model is considered as a relatively easy procedure through sparing the tibial nerve and cutting the common peroneal and sural nerves, it requires the ligation of the sural and common peroneal nerves separately with potential risk of stretching the sciatic or tibial nerves." (Line 52-55)
I do not quite understand the advantage of manipulating the common peroneal and the tibial nerve over manipulating the common peroneal and the sural nerve. Of course, the authors were able to use one ligature instead of two separated ones, resulting in a time advantage. However, the risk of stretching the unaffected nerve (tibial or sural) or the sciatic nerve, stays the same.

With our explanation above, we hope the reviewer has better understand on the advantage in sparing tibial nerve.

#3 "Based on this anatomical feature, we developed a modified mouse SNI surgery procedure to ligate the common peroneal and sural nerves together with only one nerve-ligation and one nerve-cutting, which results in shortened procedure-time with avoidance of the potential sciatic nerve stretch or tibial nerve injury." (Line 60-63)
The authors have written in the introduction that they "present a modified SNI surgery method to damage both common peroneal and tibial nerves with only one ligation and one cut with much shorter procedure time" (line 28-30). However, the introduction indicates that the authors developed a surgery method ligating the common peroneal and sural nerves. The authors need to clarify what surgery has been performed.
Furthermore, the authors might want to refer to e.g. to Decosterd and Woolf, 2000 (DOI:10.1016/S0304-3959(00)00276-1), who established an SNI-model manipulating the common peroneal and the tibial nerve, or Shields et al., 2003 (5), introducing a method to manipulate the common peroneal and the sural nerve, and Cichon et al., 2018 (7), who published a detailed method paper describing the application of one ligature and one nerve-cutting for a SNI model in mice.

We thank the reviewer for picking up the mistake. In the revise manuscript we have corrected the sentence to "present a modified SNI surgery method to damage both common peroneal and sural nerves with only one ligation and one cut with much shorter procedure time". Cichon paper has been cited in the manuscript.

#4 Protocol 2. Modified SNI surgery

The description of the modified SNI surgery is again appears to be contradictory to the abstract and needs to be revised.
Moreover, a description of the surgery procedure applied for the "traditional group" is missing.
In general, detailed information about the used materials, reagents and equipment would improve the reproducibility of the presented surgical method.

We thank the reviewer for picking up the mistake and we have corrected in the abstract. We also added the traditional procedure in the protocol.

#5 "2.7 Cut nerve at distal part within 2-4 mm to the ligation with a pair of micro scissors. To note, the tibial nerve remains untouched during the whole procedure." (Line 106 - 107)
Does the procedure involve the formation of a nerve defect (size?), as usually applied for SNI models? Or did the authors perform a simple axotomy?

A small part of the ligated nerve was removed to prevent nerve regeneration between proximal and distal parts.

#6 Protocol 3. von Frey assessment for mechanical threshold (Line 109 - 121)
The authors did not make clear, whether the Von Frey assessment involved an acclimatization of the animals 1-2 weeks ahead of testing, as suggested by one of the cited publications: Wilson & Mogli, 2001 and in accordance with the ARRIVE guidelines for animal (9).
Additionally, the authors might explain, why only the withdrawal threshold and not the relative frequency of paw withdrawal was recorded and evaluated.

In this study, we stimulated the mid-plantar of the hind paw with von Frey filaments with up-down paradigm from Chaplan et al (citation 12). As the reviewer pointed out, von-Frey assessment involved at least 6 days of acclimatization of mice to the testing room environment and testing materials in this study. Place the mice in the clear plastic cylinders on an elevated wire mesh grid for 1 hour of habituation. White papers are placed between each cylinder to prevent any visual cue from each testing animal. During this period, 60min habituation was performed every two days, and the first von Frey monofilaments was applied under the middle of the hindpaw after the last 60min habituation. In addition, we further have added data of "percent response" method to assess the mechanical hypersensitivity in the results (Figure 5b).

#7 Protocol 4. Representative Results 4.1. The comparison of procedure time between modified and traditional methods (Line 122-129)
Comparing the animal numbers (4.1. procedure time traditional: n=5, modified: n=5; 4.2. von Frey traditional: n=9, modified: n=14), 4 animals of the traditional method and 9 animals undergoing the modified method were excluded from the analysis. Kindly provide some rational behind this.
However, the procedure time of a surgery should not be a criterion for a surgery method, as long as the animal does not have a clear benefit (e.g. significant reduction of anesthesia). Instead, the reproducibility of the method and the stability of neuropathic pain behavior should be taken into consideration.

Mice in Figure 4 were used to observe the difference of procedure time between the two methods. They

were not used for von-Frey assessment. We do not agree with the reviewer that the "the procedure time of a surgery should not be a criterion for a surgery method". Quite often researcher in neuropathic pain field need to do surgery in many animals for any single experiment, therefore cutting procedure time in half can save substantial amount of time.

#8 "No difference of von Frey assessment was observed in two groups at baseline (1.05±0.10 vs. 0.96±0.13g, p=0.9405)." (Line 131-132)
Please provide some information about the baseline measurement in the protocol.

The baseline results are clearly shown in Figure 5a and 5b.

#9 "Compared with traditional method (n=9), the modified method (n=14) induced similar mechanical hypersensitivity on the ipsilateral side from post-operative 1 day to post-operative 14 days"
How was the number of animals calculated? Please report sample size calculation as recommended in the ARRIVE guidelines for reporting animal research (doi: 10.1371/journal.pbio.3000410).

We need to point out that our current method is the modification of the existing method we have used and published for many years. We chose the sample size based on our previous experience.

#10 "Indeed, we demonstrate that no nerve regeneration was observed 14 days after modified SNI; […]." (Line 151-153)
Fourteen days observation time for peripheral nerve regeneration is rather short. Spontaneous regeneration after axotomy usually appears after 4 weeks. Therefore, the removal of nerve stumps appears to not be necessary. This point should be discussed, although figure 3D suggests that the nerve stumps are not located close enough for spontaneous regeneration.

Although 4 weeks follow-up, as suggested by the reviewer, could be more powerful to support our result, no nerve regeneration observed 14 days after modified SNI should be taken into account, because the axon regeneration proceeds at a rate of 1-3 mm/d. (Sulaiman, W. & Gordon, T. Neurobiology of peripheral nerve injury, regeneration, and functional recovery: from bench top research to bedside application. Ochsner J. 13 (1), 100-108, (2013).)

#11 Discussion (Line 146 following)
The authors carved out the benefits of their method regarding the required time and the surgery procedure itself. A discussion of the efficiency of the protocol, reflected by the stability of neuropathic pain, would have strengthened this section. For this purpose, the assessment of neuropathic pain should not only include mechanical allodynia but e.g. cold allodynia, heat hyperalgesia and spontaneous pain. I would have furthermore welcomed it if the authors would have discussed the translatability of the model into rats, especially focusing on automutilation behavior after sciatic nerve injury in rats.

Again, our current method is the modification of the existing SNIt method we have used and published for many years. In this model, there is no heat hypersensitivity or automutilation.

#12 Figure 1
The schematic drawing of the surgical area might be misleading and should be reviewed. For example: the figure indicates that the authors cut through the femoral biceps muscle and did not perform a blunt dissection like reported in the protocol. The labeled muscles probably correspond to the medial gluteal muscle or the femoral quadriceps muscle and the cranial tibial muscle (?).

*In the schematic drawing of the surgical, the muscle labeled BFM is biceps femoris muscle (Cichon, J., Sun, L. & Yang, G. Spared Nerve Injury Model of Neuropathic Pain in Mice. Bio Protoc. **8** (6), (2018)); and the muscle labeled GM is gastrocnemius muscle (Rupp, A. et al. Electrophysiologic assessment of sciatic nerve regeneration in the rat: surrounding limb muscles feature strongly in recordings from the gastrocnemius muscle. J Neurosci Methods. 166 (2), 266-277, (2007)).*

#13 Figure 3
Did the pictures originate from another study or did the authors performed surgery on animals they forgot to report in the manuscript? I am confused about the white fur as only C57BL/6 mice were reported to be used in this study (see line 67).
However, it would be interesting to know if figure 3A-C originate from the unaffected, contralateral side, or sham operated animals. The exemplary pictures are probably unfavourable, as they might give the reader the impression that the sural nerve is damaged and the ligation (figure 3D) only includes the common peroneal nerve.

The mouse used in Fig. 3 was purely for demonstration and was not used for testing the behavior. The exemplary pictures are right to the point to show the damage of sural and common peroneal nerves.

#14 Figure 4 & 5
Please provide details of the statistical methods used for each analysis, including the used program and the confirmation of normal distribution. Furthermore, the number of animals included in both analysis could be indicated in the figure legend.

We have revised the manuscript as suggested.

Minor Concerns:
Do not apply.

Reviewer #3:
Manuscript Summary:
The authors developed a simpler technique for the mouse sparing nerve injury (SNI) model that only requires one ligation and one incision to damage both the common peroneal and sural nerves. it is simplified relative to existing spared nerve injury model.

Major Concerns:
1. In page 7, line 120, Authors stated "The von Frey assessments were performed on the pre-surgery 1 day, and the days 1, 3, 7, 14 days after the procedure. but Figure 5 data shows time points "days 1, 3, 5, 7 and 14". The figure shows day 5 data but this time point was not mentioned in the procedure.

We have added day 5 in the procedure as suggested.

2. There is no mention of post-operative care in the protocol.

We have added the post-operative care in the protocol (2.9) as suggested.

3. When mechanical hypersensitivity was assessed in nine to fourteen animals, why did the authors present procedure time in only five animals?

Mice in Figure 4 were used to observe the difference of procedure time between the two methods. They were not used for von-Frey assessment.

Minor Concerns:
1. The word "modified" must be included in the title since there is a major change in method. title could be such as "Modified Spared nerve injury" instead of "Simplified Spared nerve injury"

The title has been revised as suggested.

2. It would have been preferable if the authors had evaluated at least two parameters in order to quantify neuropathic pain.

We further added data of "percent response" method to assess the mechanical hypersensitivity (Fig. 5b).

3.The authors have not included any information on the equipment (whether dynamic plantar aesthesiometer or manual von frey filaments) used to test mechanical hypersensitivity

We have revised the protocol as suggested.

Reviewer #4:
Manuscript Summary:
He et al. provide a modified method for mouse SNI model that involves one cut and ligation. The authors state that the procedure is much shorter than the traditional method and this method reduces the potential risk of stretching sciatic or tibial nerves. Overall, I think this method could be a nice addition to the host of neuropathic pain models that already exist in the literature. However, the conclusions as currently stated in this article lower my enthusiasm for the work. The authors show a reduction in surgical time from 5 min to 2.5 min. Having done many SNI and sham operations I don't think the surgical time was a major concern. More importantly, they also state their technique has reduced "potential stretching of sciatic or tibial nerve" without any evidence to support such a claim. If the authors could provide additional evidence to show this point, the work would be enhanced.

Major Concerns:
In abstract the authors that the traditional SNI mouse model requires ligating and cutting the common peroneal and sural nerves, leaving the tibial intact. My understanding is that the sural is left intact and

the lateral aspect of the hindlimb is probed for withdrawal response. Which paper are the authors referencing?

It is confusing in SNI neuropathic pain model because there are two types of SNI models. The first one was initially developed by Decosterd and Woolf, 2000 in rat (added as citation 5), in which common peroneal and tibial nerves are injured. This method, which we defined as "SNIs" in the revised manuscript because the sural nerve remains intact, was eventually applied in mouse (citation 6). The second one was initially developed in Dr. Basbaum's lab in mouse and is normally used in our lab, in which peroneal and the sural nerves are injured (citation 7&8). In the revised manuscript we defined the second method as "SNIt" because the tibial nerve remains intact. Mouse sciatic nerve has three major branches, the tibial nerve, the common peroneal nerve, and the sural nerve. Both tibial and common peroneal nerves contain both sensory and motor fibers, and the sural nerve is a pure sensory nerve. As the result, the SNIt model developed by Dr. Basbaum's lab is superior that the SNIs model developed in Woolf's lab because the SNIt model has much less motor deficit. Our current manuscript is about the modification of SNIt model.

Last sentence of introduction. "avoidance of the potential sciatic nerve stretch or tibial nerve injury." This is a strong statement without any evidence to suggest stretching is involved in their technique or the traditional SNI procedure.

We have removed the sentence as suggested.

Protocol. Do the authors use an analgesic at the time of SNI? Local anesthetic, NSAID?

We have previously compared the SNI animals with and without local anesthetic and NSAID at the time of SNI and found that local anesthetic and NSAID significantly reduced neuropathic pain behavior after SNI. As the result, we did not use local anesthetic or NSAID in this protocol.

Figure 4 title ."The modified method is superior to the traditional SNI to perform." Just by measuring time does not support the claim of a superior technique to perform. This should just state that the authors report faster procedure time as compared to conditional SNI under comparable levels of training by the experimenter.

The title of the figure has been revised as suggested.

Results section 4.1. "The procedure time from the beginning of cutting skin to the end of closing was recorded in 5 mice of modified approach and 5 mice of traditional approach." This seems like a small n number to support the major conclusion of the work. Why are there less mice here than in Figure 5?

Mice in Figure 4 were used to observe the difference of procedure time between the two methods. They were not used for von-Frey assessment.

No controls performed.

The control was the original SNIt model.

Minor Concerns:
First sentence of abstract. SNI is useful for both rodent (rat and mouse) studies of neuropathic pain not just mouse.

We have revised the abstract as suggested.

Introduction. "the greatest and most reproducible hypersensitivity area is produced in the middle of the hip paw innervated by the tibial branch of the sciatic nerve." Is this hindpaw?

Yes. We have revised the introduction as suggested.

Figure 4 y axis needs space between procedure time and sec.

We have revised the figure as suggested.

Figure 5 y axis needs space between mechanical threshold and (g)

We have revised the figure as suggested.