

To Nam Nguyen, PhD
Manager of Review
JoVE

Würzburg, 11th of May 2021

Dear Dr. Nguyen,

We are thankful for your editorial help and the constructive criticism of the expert reviewers that have helped to improve the revised version of the manuscript “Multifactorial assessment of motor behavior in rats after unilateral sciatic nerve crush injury”. We have included changes to the manuscript (changes are written in **bold** with underlining), the figure 3, and the video. In addition, we would like to reply to the editor’s and reviewers’ comments (the editor’s and the reviewers’ comments are in *italic*) as follows:

Editorial and production comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We performed a proofreading of the manuscript and corrected any spelling and grammar issues.

2. Please mention how proper anesthetization is confirmed.

We added the information about the confirmation of proper anesthesia in the manuscript. “Control of the anesthesia depth by pinching the interdigital webbing of the hind feet. The absence of withdrawal reflexes indicates an adequate anesthesia.” (line 88-89)

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Personal pronouns were removed in the protocol section. (line 39, 179, 180, 220, 231, 307, 329, 411)

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible.

We revised actions of the protocol section in the imperative tense. (line 108, 152-155, 162-164, 200-201, 232-233, 288, 345, 450, 486-488)

5. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

We moved action items, which are not absolutely necessary in the protocol section to the discussion section. (line 642-657)

6. Please specify all experimental parameters. Be more explicit on the setup of the assays and the positioning of the cameras, etc.

We specified the information in our manuscript (line 152-155, 226-228, 336, 486-488)

7. Please spell out the journal titles in the references.

We now spelt out the full name of the journal titles in the references.

Changes to be made by the Author(s) regarding the video:

1. Title Cards:

- *Please capitalize the first letter of every important word in your title.*

We capitalized the first letters of every important word in our title cards.

2. Video Editing Content:

The Following segments with vertical video are not ideal for JoVE videos, we recommend you reshoot this segment in landscape mode:

- *04:22*
- *04:43*
- *05:40*
- *06:13*
- *06:29*

We reshot the mentioned segment in landscape mode.

- *01:50 - 01:58 Consider removing that's 8 seconds of ""dead air"" Shot which is not necessary to show the Audience.*

We removed the mentioned segment.

- *03:09 there is a pop-up image in the top right of the frame that doesn't remain on screen long enough for us to acknowledge and understand it. I would suggest to them that they extend the duration of this overlay so it can be seen properly.*

We extend the duration of the mentioned segment.

3. Audio Editing and Pacing:

- *Audio Levels are quite Low and not Balanced. Please ensure audio level peaks average around -9 dB.*

We improved the audio levels and ensured the correct audio level.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

With their manuscript the authors provide a protocol on how to assess motor behavior and functional deficits in rats with unilateral sciatic nerve crush injury.

The protocol is comprehensive, detailed and valuable to be published as it provides researchers interested in the topic with additional information on how to perform multifactorial assessment of functional deficits and motor behavior.

I have no major concerns regarding the manuscript and support its publication, although in my opinion some minor issues should be amended.

Major Concerns:

None

Minor Concerns:

1. Lines 39-40: The authors state that rodents were excellent model organisms to deepen understanding of human disease. Besides that I miss a reference to support this statement I also don't agree 100% with it. For sure rodents are excellent model organisms of human disease, but I would appreciate a more critical discussion of their use in translational research. This is especially relevant regarding translational research in peripheral nerve repair, in which rodent models have some clear limitations. A study of interest in this regard is: <https://www.ncbi.nlm.nih.gov/pubmed/26296419>

We apologize for our not clearly defined statement and thank for the reference regarding the nerve regeneration in rat models. Our statement “Rodents are excellent model organisms to deepen the understanding of human diseases by testing hypotheses on multiple biological levels.” was referring to the possibility of a multiscale characterization of rodent models, which includes the level of molecular biology, cellularity, microcircuitry, and large-scale network. However, we agree that the critical discussion about the translational aspect of regeneration processes is important to mention in our manuscript. We therefore added following statement and included the recommended reference: “Even though the translational aspect of nerve regeneration from rat to human has to be regarded critically. the...” (line 49-50). In addition, we selected two additional references to support the statement of rodents being excellent model organisms. (line 39)

Iannaccone, P. M. & Jacob, H. J. Rats! Disease Models & Mechanisms. 2 (5-6), 206-210, (2009).

Phifer-Rixey, M. & Nachman, M. W. Insights into mammalian biology from the wild house mouse *Mus musculus*. Elife. 4, (2015).

2. Lines 46-74: I would recommend amending the statement, since it reads as the sciatic nerve would receive motor and sensory innervation. However, it does provide sensory and motor innervation due to its nature as a mixed nerve. I completely understand what the authors want to express, but maybe the authors could rephrase this sentence.

We understand that this statement is confusing. We rephrased our statement to: “The sciatic nerve is the largest nerve in the human body with motor as well as sensory fibers.” (line 46)

3. Lines 51-52: Please rephrase this statement, it should read something like that "in case of a crush injury the nerve is not completely transected".

We rephrased the statement “In contrast to neurotmesis, that is the most severe form of nerve injury, the sciatic nerve crush injury does not transect the whole nerve completely.” to “In case of a crush injury the nerve is not completely transected.” (line 52)

4. Line 53: Axonotmesis injury does not cause a conduction block, which is the pathognomonic finding in case of neurapraxia. Please amend this statement accordingly.

At early time points after axonotmesis, CMAP and conduction data cannot be distinguished from neurapraxia. Distal nerve segments are still excitable and show normal conduction. However, the proximal nerve segments show smaller or absent CMAP and a conduction block. A discrimination between axonotmesis and neurapraxia is possible only after processes of Wallerian degeneration, which takes about nine days after injury (Reference: Robinson, L. R. Traumatic injury to peripheral nerves. *Muscle Nerve*. **23** (6), 863-873, (2000)). In our study, we measured the nerve conduction velocity directly after nerve crush injury and found a complete conduction block of the proximal part of the injured nerve, while the distal part of the sciatic nerve was unaffected (Reference: Knorr, S. et al. The evolution of dystonia-like movements in TOR1A rats after transient nerve injury is accompanied by dopaminergic dysregulation and abnormal oscillatory activity of a central motor network. *Neurobiol Dis*. 10.1016/j.nbd.2021.105337 105337, (2021)). We therefore would like to keep the statement as it is. However, we would agree to change it, if the referee feels strongly about this.

5. Line 95: This should read "Search for the sciatic notch of the ilium". The sciatic nerve itself does not have a notch.

We apologize for our mistake and corrected our statement.
“Search for the sciatic notch of the ilium.” (line 97)

6. Lines 170-171: I would recommend stating the reason behind this step in the protocol (as the authors have already done in the following sections)

We added the recommended information to the protocol.
“After each trial, remove the rat gently from the testing arena and clean the setup with 0.1% acetic acid to avoid distraction by the smell of the previously recorded rat.” (line 173-174)

7. Line 185: Although the information regarding the Illuminated footprints technology is correct, I would recommend reflecting if it is absolutely necessary to state this here, it reads a little bit like an advertisement.

We totally understand the concerns of the referee and therefore deleted the sentence: “The CatWalk XT system is based on the principle of Illuminated Footprints™ technology from Noldus.”

8. Lines 215-218: In my opinion it would be preferable to indicate that the chosen settings, especially the Maximum run variation, are somehow arbitrary. The authors could indicate that these settings were chosen because they have worked well for them.

We changed our specific statement to a more general statement.
Previous: “Set “Minimum run duration” to 0.5 sec, “Maximum run duration” to 12.0 sec, “Minimum number of compliant runs to acquire” to three, and checkmark the box of “Use

maximum allowed speed variation” and set it to 50%. The run criteria can be ignored for the first four to five days of training.”

New: “Set appropriate values for “Minimum run duration”, “Maximum run duration”, and “Minimum number of compliant runs to acquire”, which are specific for every research project. Checkmark the box of “Use maximum allowed speed variation” and set the value. The “Run criteria” can be ignored for the first four to five days of training.” (line 220-224)

9. Lines 221-222: It is true that by placing the camera closer to the walkway the resolution can be improved. But on the other hand, this also reduces the length of the walkway which can be recorded. I would recommend indicating that a compromise between these two variables must be chosen.

We thank the reviewer for this comment and included this recommendation in the manuscript. Previous: “The position should be as close as possible to achieve optimal resolution of the recorded paws.”

New: “Find the optimal camera position to achieve an appropriate length of the walkway and the best resolution of the recorded paws simultaneously.” (line 226-228)

10. Lines 244-251: Maye the authors could include that it is not obligatory to use the goal box. It is also possible to place a normal cage at the end of the walkway, which allows the rats to enter their "home territory" after each run.

We included the recommendation in the manuscript.

“Furthermore, it is also possible to remove the goal box from the CatWalk system and to place the rat home cage at the end of the walkway, which allows the rat to enter their "home territory" after each run.” (line 652-654)

11. Line 261: I believe this should read "experimental" settings.

We apologize for our mistake and corrected it in our manuscript.

Reviewer #2:

This paper describes various methods to analyze motor function of rats after sciatic nerve injury. All procedures were well organized and demonstrated with clear images.

For the readers, I recommend to add some information as below:

Are there good correlations between the data of various methods of open field test, CatWalk, beam walking task, and the ladder rung walking task? Since all methods cannot be tried at the same time, it is better to provide the rationale which method(s) should be chosen.

We performed all four behavioral tests within two days by using the same rats.

For new studies with an unclear animal phenotype, we recommend to perform a pilot experiment with a behavior test battery. Behavioral tests with a good characterization of the animal phenotype should be used for further experiments of the specific study. A general recommendation for the use of a specific behavior test cannot be made, because it is dependent on your scientific research question.

We added a statement in the manuscript regarding your concern.

“The same group set of animals were used in all described behavioral tests. A maximum of two different behavioral tests per day were performed for each animal. If behavioral tests are

performed in regular intervals, pay attention of a comparable procedure, like performing the test in the same animal order and at the same time of the day.” (line 630-634)

I cannot follow the reproducibility of the sciatic nerve injury. It is good to use a non-serrated clamp, but how is the clamp pressure controlled? It is quite important because the condition would significantly affect the severity and the restorative capacity of the movement disorders. Please state more in details.

We use a clamp, which is commercially available and manufactured by a standardized process. Furthermore, we pay attention to order always the same clamp (company and catalog number), to use the same parameters (duration, position of the ratchet), to use the clamp only for a nerve crush, to handle the clamp with care during and after the crush surgery. We added following statement to the section of a consistent crush technique “The exclusive use of the clamp for the crush injury and handling of the clamp with care improves consistency” (line 621-622)

Are these methods available for mice as well? Indeed, mice are often used for the motor function analyses especially when we need transgenic animals. Please provide comments in the text.

The nerve crush injury as well as all described behavioral tests by using can also be used for mice. However, the size of setups and settings need to be adjusted for mice.

We added a statement in the manuscript:

“It is important to mention that the nerve crush injury as well as all described behavioral tests can easily translated to mice, by adapting the settings and sizes of the setups. The use of mice as a model organism has the beneficial effect that transgenic models for many human diseases exist.” (line 689-692)

Reviewer #3:

Manuscript Summary:

This methodological study aimed to achieve consistent, reproducible, and comparable results after a sciatic nerve crush injury. A standardized method for inducing the nerve crush is essential, in addition to a standardized phenotypical characterization. This protocol was well described and executed in explicit detail to perform a sciatic nerve crush injury and provides a behavioral test battery to assess motor deficits in rats that include the open field test, the CatWalk XT gait analysis, beam walking task, and the ladder rung walking task. The manuscript is well written to explain all the detailed steps involved in the performance of the four procedures outline. This is a precious methodological study.

Major Concerns:

none

Minor Concerns:

Methodology;

What is the number of animals used per group?

We included the number of the used animals in our manuscript.

“... in naïve wildtype (wt) rats (n= 8-9) and wt rats five weeks after unilateral sciatic nerve crush injury (n= 10).” (line 64-65)

1.13. Explain why using Rimadyl.

We added an explanation for the use of Rimadyl into the manuscript.

“Apply Rimadyl according to the GV-SOLAS guidelines (5 mg/kg body weight, subcutaneous injection) for postoperative pain relief every 24 hours after surgery for two days.” (line 126-127)

2.2.2.1.7. Elaborate more on how to calibrate the walkway.

We added a more detailed description of the procedure “Calibrate the walkway”.

“Click the “Calibrate Walkway” icon. Position a rectangular calibration sheet measuring 20 x 10 cm in the middle of the walkway. Adapt the size of the white rectangle to the calibration sheet. Click “OK”.” (line 242-244)

2.2.2.1.8. Clarify how to snap a background image.

We clarified how to snap a background in the protocol.

“Check beforehand that the walkway is clean and empty. Click the “Snap Background” button to generate a background image.” (line 246-247)

2.2.4.4. this step needs more elaboration, especially for the trainees/beginners using the Catwalk apparatus.

We added a more precise description into the manuscript.

“Move the video to a position, which has to be reviewed manually. For correction of wrong labeled paw prints, select the rectangle of the specific paw print, click “Reset”, select the same rectangle again, and assign the correct label from the list. For labeling non-detected paw prints, draw a rectangle around the non-detected paw, click “Add Print”, select the new generated rectangle, and assign the correct label from the list. In case the software labeled nose or body prints automatically, select the rectangle of the specific label, and click “Remove Print”.” (line 297-303)

2.3. define the difference between motor coordination and motor balance.

“Motor balance is the ability for postural control during body movements.”

“Motor coordination is the ability to coordinate muscle activation from multiple body parts, this includes in innerlimb coordination as well as limb position coordination.”

The definitions were added into the manuscript.

“Gait deficits can be determined by the beam walking task. The focus of the beam walking task in this specific research topic will be the analysis of motor coordination, defined as the ability to coordinate muscle activation from multiple body parts, and not the assessment of motor balance, defined as the ability for postural control during body movements.” (line 328-331)

2.3.3.7. line 401-402 - Explain if the experiment is for 5 weeks, why the testing continues for 6 weeks?

We apologize for the error, it should be written “five”. We changed the error in the manuscript. (line 400)

2.4.4.4. line 458 - "Take the first form" should be "Take the first from."

We apologize for our mistake and corrected it in the manuscript. (line 456)

Results:

Although the representative results of the 5 min OFT shows that the nerve crush injury five weeks post-surgery has no effect on the locomotor activity, this test may be precious and suitable for early animal motor assessment during the first and second weeks post-crush nerve injury particularly if the experiment includes treatment group(s).

We also analyzed these animals at an earlier time point (week two post surgery) without detecting a significantly difference on the locomotor activity. We also think that the locomotor activity can be affected in treatment groups or transgenic animals after a sciatic nerve crush injury.

We included a statement in our manuscript:

“Although not every analysis yielded significant differences in this study, consider that an inclusion of genetically modified animals or treatment groups could produce valuable data that can distinguish between groups from the same behavioral tests.” (line 658-660)

Discussion:

Did the Authors use different group sets for each test? This point is not clear. Or did the Authors subjected the TWO groups to ALL different tests? This point should be clarified and explained.

We used the same group set for all behavioral tests and performed maximum two different behavioral tests per day. If behavioral tests are performed in regular intervals, we always pay a lot of attention of a comparable procedure, like performing the test always at the same time of the day etc.

We added a statement into the manuscript.

“The same group set of animals were used in all described behavioral tests. A maximum of two different behavioral tests per day were performed for each animal. If behavioral tests are performed in regular intervals, pay attention of a comparable procedure, like performing the test in the same animal order and at the same time of the day.” (line 630-634)

It is not clear why excluding the balance assessment in the beam walk task test. Balance is the function of sensory and motor coordination functions that is very helpful in sciatic nerve injury recovery assessment since the sciatic nerve is a mixed (sensory and motor) nerve.

Our research group is working in field of movement disorders and we translated the nerve crush injury to our dystonia research. The sciatic nerve crush injury is used as an environmental trigger in dystonia predisposed subjects to elicit dystonia-like movements. The dystonic phenotype should be assessed due to the beam walking task. In dystonia patients abnormal postures and movements are observed, however no obvious abnormalities in motor balance. Consequently, we adapted the assessment of the beam walking task to our research interest and excluded the motor balance from the analysis.

Vet Review

Were animals used humanely and was the appropriate anesthesia or analgesia provided for potentially painful procedures?

Please provide additional comment, if necessary.

1. Please be specific in your comments. If possible, divide your comments into 2 categories:

b) Improvement (recommendations) - for minor deviations, missing parts, etc....

I believe that the researchers took appropriate steps to ensure the welfare and well-being of the animals, including using appropriate anesthesia, and following accepted aseptic surgical techniques. The only veterinary related question would be why they did not provide post-operative analgesia (pain relief). From the perspective of US regulations, they would have to justify withholding analgesics postoperatively, and I don't see where in this case it would be justified.

We would like to specify the used analgesia for the nerve crush injury in rats. We provide ~1 h before the surgery pre-operative (preemptive) analgesia (with Rimadyl s.c.) and post-operative analgesia after the surgery every 24 h for a minimum of two days. In case we find signs of postoperative pain in the animals after two days, analgesia treatment will be prolonged. In the manuscript, we mentioned the post-operative analgesia in the section "Sciatic nerve crush injury": "Apply Rimadyl according to the GV-SOLAS guidelines (5 mg/kg body weight, subcutaneous injection) for postoperative pain relief every 24 hours after surgery for two days." (line 126-127).

We also added in the discussion section a recommendation about pain relievers: "Therefore, a careful nerve preparation as well as an administration of a pain reliever for a minimum of two days is recommended." (line 624-626)

From a research design perspective, they indicate that some of the test should be done in darkened or low light rooms. Since rats are nocturnal, and their activity is considered "normal" during dark hours, if they are removed from lighted rooms and immediately placed in a darkened room, they will not have had sufficient time to acclimatize to the "new" photoperiod. Ideally, the rats should be maintained under reverse light cycle, so that their night hours (dark period) occurs during the normal human work day. Most test were recommended to be in darkened rooms, but the beam crossing test was done in normal room light. This introduces a variable between this and the other tests.

We totally agree that a reversed light cycle would be better for the rats when performing behavioral assessment, because of their natural activity. However, for some of the behavior tests, high-resolution videos are needed for the analysis. However, light is essential for high resolution videos. The existing night cameras are very expensive and the quality of the videos are not as good as recordings with good illumination. Furthermore, the behavioral test performed with light are based on forced motor activity and not on the spontaneous activity, like the open field test. Therefore, the environment has not a strong effect on these data. Additionally, for other behavioral test, a dark environment is needed, like for the CatWalk

gait analysis. We made the compromise that we don't switch the light cycle, because also other researchers have to perform other behavioral test by using the light. Therefore, we let the rats acclimate in the testing room under testing conditions for at least 30 min.

We mentioned this aspect in the manuscript.

“A further important aspect for behavioral analysis is the day-night cycle of rats. Consider a reversed day-night cycle to obtain more natural and higher levels of activity at the day cycle (dark cycle). This has to be considered especially for measurement of spontaneous behavior, like the OFT. In this experiment, a reversed day-night cycle could not be implemented, but an adequate acclimatization to the testing conditions was ensured. A perfect illumination is essential for high-resolution videos for the beam walking task and the ladder rung walking task. This high video quality cannot be reached when performing experiments in the dark.”
(line 634-641)