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Multifactorial assessment of motor behavior in rats after unilateral sciatic nerve crush injury

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TITLE:

Multifactorial Assessment of Motor Behavior in Rats after Unilateral Sciatic Nerve Crush Injury

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KEYWORDS:

neuroscience, motor behavior, open field test, CatWalk XT gait analysis, beam walking task, ladder rung walking task, sciatic nerve crush injury, dystonia, DYT-TOR1A, rat

SUMMARY:

We provide a protocol for the assessment of motor behavior via a behavioral test battery in rats after sciatic nerve crush injury.

ABSTRACT:

The induction of a peripheral nerve injury is a widely used method in neuroscience for the assessment of repair and pain mechanisms among others. In addition, in the research field of movement disorders, sciatic crush injury has been employed to trigger a dystonia-like phenotype in genetically predisposed DYT-TOR1A rodent models of dystonia. 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. Attention must be paid not only to the specific assortment of behavioral tests, but also to the technical requirements, the correct execution and consecutive data analysis. This protocol describes in detail how to perform a sciatic nerve crush injury and provides a behavioral test battery for the assessment of motor deficits in rats that includes the open field test, the CatWalk XT gait analysis, the beam walking task, and the ladder rung walking task.

INTRODUCTION:

Rodents are excellent model organisms to deepen the understanding of human diseases[1,2] by testing hypotheses on multiple biological levels. One fundamental biological level for the characterization of rodent models is the phenotype level, measured by behavioral assessments. Depending on the animal model and the scientific research question, the selection of a powerful and reliable behavioral test battery is essential to cover a wide range of behavioral aspects such as for animal models of Parkinson's disease and dystonia[3-6].

The sciatic nerve is the largest nerve in the human body with motor as well as sensory fibers. Injuries of the sciatic nerve can result easily from a variety of events such as traffic accidents and surgeries[7,8]. Therefore, research activities using rodent models with sciatic nerve injuries, are of translationally relevant value. Even though the translational aspect of nerve regeneration from rat to human has to be regarded critically[9], the sciatic nerve crush injury (axonotmesis) in rodent models is a commonly used method to analyze degeneration and regeneration processes of peripheral nerves[10,11]. In case of a crush injury the nerve is not completely transected. It damages the axon, resulting in conduction block directly after crush injury followed by regenerative processes [4,12,13].

Moreover, in dystonia research, the unilateral sciatic nerve crush injury is an established method to trigger dystonia-like movements (DLM) in genetically predisposed dystonia rodent models, which do not show DLM per se[4,14]. It is assumed that the peripheral nerve trauma disturbs the sensorimotor integration by affecting the sciatic nerve fibers, which are responsible for motor and sensory functions[15].

We here provide a detailed description for a standardized crush injury of the sciatic nerve and a battery of motor behavior assessments that is composed of the open field test (OFT), CatWalk XT gait analysis, beam walking task and ladder rung walking task in naïve wild type (wt) rats (n= 8-9) and wt rats five weeks after unilateral sciatic nerve crush injury (n= 10). The OFT provides information about the general locomotor activity, while a detailed gait analysis is achieved by the automated gait analysis system CatWalk XT. The beam walking task is used to assess the motor coordination by evaluating the time to cross the beam and the number of foot placement errors. For gait performance analysis the ladder rung walking task gives information about foot or paw placement and errors on a horizontal ladder rung apparatus with a constant but irregular rung pattern.

PROTOCOL:

All animal experiments were approved by the local authorities at the Regierung von Unterfranken (Würzburg, Germany) and performed according to applicable international, national, and/or institutional guidelines for care and use of animals.

1. Sciatic nerve crush injury

NOTE: Maintain a sterile environment during the whole surgical procedure. Set up the surgery table with the necessary equipment.

1.1. Deeply anesthetize the rat in a closed cabinet with isoflurane 3.0% in O_2 (2 L/min). Remove the rat from the cabinet. Shave an extensive area of the right hindlimb.

1.2. Position the rat into the anesthesia mask and continue deep anesthesia with isoflurane 2.0% in O_2 (2 L/min). Control the anesthesia depth by pinching the interdigital webbing of the hind

feet. The absence of withdrawal reflexes indicates an adequate anesthesia.

1.3. Fix the torso and both hindlimbs of the rat with tape. Place both hindlimbs in a symmetrical and outstretched position by turning the paw flat onto the surgery table.

1.4. Apply ophthalmic ointment on eyes to prevent dry eyes. Disinfect the skin of the shaved area with an antiseptic.

1.5. Search for the sciatic notch of the ilium.

1.6. Make a skin incision from the sciatic notch into the direction of the paw with a scalpel. The skin incision should be as small as possible (approximately 1 to 2 cm).

1.7. If the hindlimbs are fixed and the skin incision is performed correctly, a cavity in the fascial plane between the gluteus maximus muscle and biceps femoris muscle can be seen that resembles a “white line”. Insert closed super-fine hemostatic forceps (No. 5) into the cavity and spread the forceps. The fascial plane should open up without injuring any muscle tissue.

1.8. Place the rubber band retractors underneath the muscles, to keep the skin incision open.

1.9. Gently remove any surrounding tissue and blood vessels from the sciatic nerve until the nerve is completely exposed. It is important to not stretch or pull the nerve during the whole procedure.

1.10. Crush the sciatic nerve with a non-serrated clamp (ultra-fine hemostat) with constant and reproducible pressure. For this, open the clamp, place the nerve onto the bottom jaw of the clamp and close the clamp by locking it into the first position for three times ten seconds. The position of the sciatic nerve crush is located close to the sciatic notch, proximal to the division site of the main sciatic nerve bundle. After the crush injury, reopen the clamp carefully. The crush site of the sciatic nerve appears translucent.

1.11. Remove the rubber band retractors.

1.12. Close the fascial plane incision with resorbable 4-0 suture. Close the skin incision with body skin staples.

1.13. 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.

1.14. Remove the rat from the surgery setup. Place the rat into a clean cage without bedding on a heating plate (37 °C) until the rat is awake. Move the rat back into their clean home cage.

1.15. Remove body skin staples four to six days after surgery.

2. Open field test (OFT)

NOTE: Locomotor activity as well as behavioral activity can be analyzed by the OFT.

2.1. Setup

2.1.1. Set up the OFT (**Figure 1A**) in a dark and quiet environment. It consists of the automated video tracking system EthoVision XT (computer, software with license) and an arena measuring 58.5 cm (length) x 58.5 cm (width) x 45 cm (height) with a scratch resistant, cleanable black surface. The black surface is important to increase the contrast when tracking white animals.

2.2. Assessment

2.2.1. Place the arena and the camera into the correct position. Adjust the camera that the entire open field box with the best resolution is recorded. Perform the experiment in a dark environment. If light is needed for the setup, use a small and diffuse light to avoid light spots, reflections and shades in the arena. Ensure equal light conditions by measuring the illuminance with a lux meter in different areas of the arena.

2.2.2. Set up the EthoVision XT software. The most important settings are listed in the following. In the **Experiment Settings** choose **Live tracking** for the **Video Source** and the **Center point detection** for the **Tracked Features**. Validate the size of the arena in the **Arena Settings**. Set the start condition for data acquisition to three seconds after the rat was placed into the middle of the arena and the total run time to five minutes in **Trial Control Settings**. Choose **Static subtraction** for the **Method** in **Detection Settings.** Checkmark **Save video** for **Method** in **Acquisition Settings.**

2.2.3. Place the rat gently in the middle of the testing arena (**Figure 1B**).

2.2.4. Press the **Start Trial** button in **Acquisition Control** to start the recording.

2.2.5. During the recording, stay away from the OFT setup in order to avoid distracting the rat.

2.2.6. 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.

2.3. Data analysis

2.3.1. For the data analysis of the OFT with the EthoVision XT software, go to the **Analysis** section in the left side bar and choose **Track Visualization** under the **Results** tab (**Figure 1C**). Next, export the needed parameters to Excel. Within the software, choose a number of variables from different categories for data analysis. Important variables for this specific scientific objective are “Distance moved” and “Velocity” under the category “Distance and Time”. Perform a statistical analysis of the selected parameters (**Figure 1D**).

3. CatWalk XT gait analysis

NOTE: A gait analysis via the CatWalk XT system can help to assess many different parameters concerning the footprints, stance and gait of animal models. A glass walkway is illuminated with green light and the light scattered by the footprints of the animals is captured with a high-speed video camera, which is located underneath the walkway. The signals can be analyzed with the CatWalk XT software.

3.1. Setup

3.1.1. For gait analysis with the CatWalk XT, use the CatWalk system and the corresponding software (computer, software with license) (**Figure 2A**).

3.1.2. Perform the experiment under dark conditions, because data acquisition depends on the illumination of the walkway of the CatWalk system with green LED light. To facilitate the experimental procedure under dark conditions, illuminate the experimental room with red light.

3.1.3. Use a defined walkway that measures 65 cm in length and 7 cm in width; however, the size of the walkway depends on the size of the rats. Set up the walkway as large as possible to record as many footprints as possible for each paw.

3.1.4. Capture a minimum number of three footprints per paw for each run. When defining the length of the walkway, consider the rat's body and tail, as the start or stop signal might not be detected correctly and the runs may not be classified as compliant, if the body/tail enters or remains on the defined walkway before or after completion of the run.

3.2. Training

NOTE: Training the rats for the CatWalk system is necessary to habituate the animals to the setup and allow them to learn to cross the walkway without any interruption. Proper training provides the advantages of saving time during experimental assessment and obtaining better results. Starting the data acquisition of the CatWalk system during training sessions allows the rats to get used to the assessment conditions (noise/light).

3.2.1. Start to set up the CatWalk system.

3.2.1.1. Clean the glass walkway with distilled water and a lint-free soft cloth. At the beginning and at the end of the experiment, or in between if the glass walkway is dirty, use glass-cleaning fluid and lint-free soft cloth to clean the glass walkway. After the use of glass cleaning fluid, clear the walkway from any residues of the fluid to avoid it distracting the animal.

3.2.1.2. Choose the experimental settings. An important parameter is **Run Criteria**. 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.

3.2.1.3. Place the camera in position and adjust the focus. Find the optimal camera position to achieve an appropriate length of the walkway and the best resolution of the recorded paws simultaneously. Label the camera position on the CatWalk system to ensure identical camera placement between recordings.

3.2.1.4. Set up the detection settings by using the auto-detection for a new experiment. Be sure that all footprints can be detected with minimal background noise**.** If necessary, optimize the detection settings manually and change the Green Intensity Threshold. Use the same detection settings for the entire experiment.

3.2.1.5. Set up the corridor walls of the CatWalk system. The corridor walls should be as close as possible to the rat. Make sure the corridor walls remain parallel to the walkway.

3.2.1.6. Define the length of the walkway: Click the **Define Walkway** icon. Adjust the size of the white rectangle in length and width, according to the specific research project. Click **OK**.

3.2.1.7. 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**.

3.2.1.8. Next, snap a background image: Check beforehand that the walkway is clean and empty. Click the **Snap Background** button to generate a background image.

3.2.2. Train animals for at least eight days before starting the actual experiment. The training on successive days is recommended.

3.2.2.1. Day 1 of training: For rats to get used to the CatWalk system, allow the animal to freely explore the walkway and the goal box. Let the rats practice crossing the walkway and enter the goal box. Pick up the rat at the end of the walkway or in the goal box and bring the rat back to the starting point of the walkway. Five runs are recommended for the first day of training without need for compliance to the experimental settings.

3.2.2.2. Day 2 of training: Rats can freely explore the walkway and the goal box. Five runs are recommended without compliance to the experimental settings.

3.2.2.3. Day 3 of training: Eight runs are recommended without compliance to the experimental settings.

3.2.2.4. Day 4 of training: Ten runs are recommended without compliance to the experimental settings.

3.2.2.5. Day 5 of training: Ten runs are recommended. The experimental settings should be kept in mind. Motivate the rats to cross the walkway without any interruption.

3.2.2.6. Day 6 of training: Ten runs are recommended. The experimental settings should be kept in mind. Motivate the rats to cross the walkway without any interruption.

3.2.2.7. Day 7 of training: Ten runs are recommended. A minimum of three compliant runs should be achieved. Add more runs for animals, if they were unable to reach this goal.

3.2.2.8. Day 8 of training: Ten runs are recommended. A minimum of three compliant runs should be achieved. Add more runs for animals, if they were unable to reach this goal.

3.3. Assessment

3.3.1. According to the defined run criteria, perform three compliant runs per rat for data analysis. For the assessment, please follow steps 3.2.1. - 3.2.1.8. as described in the training section. Even if the rat reaches three compliant runs within the first three runs, carry out a minimum of six runs per session/per week for training purposes.

3.3.2. Perform at least one (training) session with six runs per week for a stable gait pattern for experiments with multiple time points. Experimental and detection settings stay consistent for the whole experiment.

3.4. Data analysis

3.4.1. For data analysis, only evaluate the compliant runs. Delete non-compliant runs.

3.4.2. Verify the Green Intensity Threshold and increase or decrease Green Intensity Threshold before classification of the paw prints if necessary. The Green Intensity Threshold must be consistent for all animals and all runs.

3.4.3. Classify paw prints automatically with the CatWalk XT software (**Figure 2B**).

3.4.4. Review paw print labels manually. Correct wrong paw print labels, add labels of non-detected paw prints, and delete noise and wrong labels manually. 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**.

3.4.5. Review the numerical results. The numerical results are displayed in an excel sheet,

showing a number of basic parameters. Choose pre-defined specific parameters, depending on the research interest and perform statistical analysis as usual (**Figure 2D**).

3.4.6. For more detailed information about each footprint, classify the toes of the hind paws. This analysis requires the **Interactive Footprint Measurements** module.

3.4.6.1. Adapt the **Green Intensity Threshold** for the **Interactive Footprint Measurements** analysis if necessary. The **Green Intensity Threshold** must be consistent for all animals and all runs.

3.4.6.2. Set the markers for the footprint analysis manually. Analyze every hind paw print in all three compliant runs. Draw a line from the center of the first toe to the center of the fifth toe to measure "Toe Spread". Draw a line from the center of the second toe to the center of the fourth toe to measure "Intermediate Toe Spread". Draw a line from the center of the third toe to the heel of the hind paw to measure "Manual Print Length" (**Figure 2C**).

3.4.6.3. Review the numerical results of the "Interactive Footprint Measurements" displayed in a separate sheet. Select specific parameters of the "Interactive Footprint Measurements" and perform statistical analysis as usual (**Figure 2E**).

4. Beam walking task

NOTE: 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.

4.1. Setup

4.1.1. For the beam walking task, use a beam, spacer, table, uniform background and a camcorder (**Figure 3A**).

4.1.2. Use a wooden beam of 90 cm in length, 1.7 cm in width and 2 cm in height. A platform of 20.5 cm in length, 15 cm in width and 2 cm in height at both ends of the beam is recommended. Use the same material for the platforms and the beam without barriers.

4.1.3. Have the distance between the beam and the table be at least 44 cm. A familiar environment such as a home cage motivates the rats to cross the beam, which can be placed at the end of the beam platform.

4.2. Training

4.2.1. Set up the beam with spacer and home cage on the table.

4.2.2. **Train animals for seven days**. The training on successive days is recommended.

4.2.2.1. Day 1 of training

4.2.2.1.1. Place all rats from one home cage on the start platform of the beam.

4.2.2.1.2. Let the rats explore the environment (platform/beam).

4.2.2.1.3. Hold one rat carefully by the tail and lead the rat to the beam by pushing the rat gently onto the beam.

4.2.2.1.4. Assist the rat in traversing the beam by holding the rat by its tail for at least two runs.

4.2.2.1.5. Let the rat traverse the beam for three more runs without assistance. Observe the rat and provide assistance if necessary. If the rat fails to traverse the beam, intercept the fall in order to avoid injuries and the development of fear to traverse the beam.

4.2.2.1.6. Continue with this procedure for all rats.

NOTE: Sometimes rats follow each other to traverse the beam, in which case no assistance is needed. However, it is important to observe the rats, intercept falls and to provide assistance if necessary.

4.2.2.2. Day 2 of training

4.2.2.2.1. Place all rats from one home cage on the start platform of the beam.

4.2.2.2.2. Let the rats traverse the beam six times.

4.2.2.2.3. If necessary, provide assistance and intercept falls.

4.2.2.3. Day 3 of training

4.2.2.3.1. Place one rat on the start platform of the beam.

4.2.2.3.2. Let the rat traverse the beam six times.

4.2.2.3.3. If necessary, provide assistance and intercept falls.

4.2.2.4. Day 4-7 of training

4.2.2.4.1. Place one rat on the start platform of the beam.

4.2.2.4.2. Let the rat traverse the beam ten times.

4.2.2.4.3. If necessary, provide assistance and intercept falls.

4.2.2.4.4. At the end of the training, the rat should traverse the beam without any interruption for at least three runs. It is permissible to gently push the rat on the start platform to trigger initiation of movement.

4.3. Assessment

4.3.1. Set up the beam with spacer and home cage on the table.

4.3.2. Place the camcorder in position, aligned in parallel to the beam with the animal in focus. The position of the camcorder should be as close as possible to the animal to achieve optimal resolution of the recorded movements. The beam and parts of both platforms should be captured by the recording.

4.3.3. Start the recording and first identify the session and the animal.

4.3.4. Place the rat on the start platform of the beam.

4.3.5. The rat should cross the beam three times without any interruption. Even if the rat reaches three compliant runs within the first three runs, carry out a minimum of six to ten runs for continuous task performance.

4.3.6. Always, observe the animal and intercept falls, if needed.

4.3.7. After the task, clean the beam and the table with 0.1% acetic acid to avoid distraction by the smell of the previously recorded rat.

NOTE: Within the first two weeks after nerve crush injury, the rats are unable to traverse the beam without assistance. Therefore, assistance has to be provided for six to eight runs in the first two weeks after nerve crush injury. From week three to week **five**, five runs are performed with assistance and ten further runs were performed without assistance.

4.4. Data analysis

4.4.1. Use the free video analysis software Kinovea for data analysis.

4.4.2. Select the video sequences of three compliant runs from the recording. For this, choose the first three compliant runs that were performed without assistance by the animal. Be consistent in the compliant run selection for all rats.

4.4.3. Define the start time point and the end time point of the selected three compliant runs

(**Figure 3D-E**). In **this** setup, the start point was labelled by a black line on the beam and the placement of the first hindlimb behind the black line defined the start time point of the run. The placement of the first hindlimb on the platform at the end of the beam defines the end time point.

4.4.4. Next, calculate the time needed for the rat to traverse the beam. Report data as latency time to cross the beam in seconds and perform statistical analysis as usual (**Figure 3B**).

4.4.5. Score the number of steps and errors from three compliant runs for both hindlimbs separately by using the zoom and slow motion function of the software. Errors include total foot slips and half foot slips. A total foot slip is defined as a foot placement that is followed by a deep slip causing a loss of contact of the affected paw with the beam (**Figure 3F**). A half slip is defined as a paw sliding off the sidewall of the beam without losing complete contact with the beam (**Figure 3G**).

4.4.6. Calculate the percentage of foot slips in relation to the number of steps to cross the beam ((number of foot slips of the limb x 100%)/ number of steps of the same limb). Present data as percentage foot slips and perform statistical analysis as usual (**Figure 3C**).

5. Ladder rung walking task

NOTE: The ladder rung walking task can assess motor function, placement of both frontlimbs and hindlimbs, and interlimb coordination.

5.1. Setup

5.1.1. Use a ladder rung apparatus, spacer, table, uniform background and camcorder for this behavioral test (**Figure 4A**). The horizontal ladder rung apparatus consists of metal rungs and clear polycarbonate sidewalls. The apparatus has a length of 119.5 cm and the width is adjusted to 7.4 cm. The walkway to be analyzed has a length of 100 cm.

5.1.2. Label the start and end point with a black line on the sidewall. Place placeholders for the rungs on the apparatus at 1 cm intervals. Arrange an irregular pattern of the rungs for the 100 cm walkway with a distance between 1 and 5 cm between the rungs. The first 10 cm at the beginning and the last 9.5 cm at the end of the apparatus, which are excluded from analysis, have a regular pattern of the rungs with a distance of 1 cm.

5.1.3. Use a distance between the walkway and the table of approximately 30 cm (**Figure 4A-B**). A goal box or a familiar environment at the end of the apparatus, like a home cage, motivates the rats to cross the ladder rung apparatus.

5.2. Training

5.2.1. Set up the ladder rung apparatus with spacer and goal box on the table.

5.2.2. **Train animals for eight days**. The training on successive days is recommended.

5.2.2.1. Day 1 of training

5.2.2.1.1. Place all rats from one home cage on the ladder rung apparatus.

5.2.2.1.2. Let the rats explore the environment (ladder rung apparatus/goal box).

5.2.2.1.3. Gently push the rats into the direction of the goal box. Assist the rats in entering the goal box. Let the rats explore the goal box for a while.

5.2.2.1.4. After all rats entered the goal box. Take the first rat from the goal box and place the rat on the start zone of the apparatus again. Continue with the same procedure for all rats of one home cage. Gently push the rats into the direction of the goal box and give assistance to enter the goal box, if needed.

5.2.2.1.5. Let the rat traverse the apparatus four times.

5.2.2.2. Day 2 of training

5.2.2.2.1. Perform the same protocol as listed for first day of training.

5.2.2.2.2. Let the rat traverse the apparatus six times.

5.2.2.3. Day 3 of training

5.2.2.3.1. Perform the same protocol as listed for first day of training.

5.2.2.3.2. Let the rat traverse the apparatus eight times.

5.2.2.4. Day 4 of training

5.2.2.4.1. Place one rat on the start of the ladder rung apparatus.

5.2.2.4.2. If the rat does not traverse the apparatus and enters the goal box voluntarily, give assistance by gently pushing the rat from behind.

5.2.2.4.3. Let the rat traverse the apparatus eight times.

5.2.2.5. Day 5-8 of training

5.2.2.5.1. Place one rat on the start of ladder rung apparatus.

5.2.2.5.2. If the rat does not traverse the apparatus and enters the goal box voluntarily, give assistance by gently pushing the rat from behind.

5.2.2.5.3. Let the rat traverse the apparatus ten times.

5.2.2.5.4. At the end of the training, the rat should be able to traverse the walkway without any interruption and assistance for a minimum of three runs. It is permissible to give the rat a gentle push in the start zone to trigger initiation of movement.

5.3. Assessment

5.3.1. Set up the ladder rung apparatus with spacer and goal box on the table.

5.3.2. Place the camcorder in position, aligned in parallel to the apparatus with the animal in focus. Position the camcorder as close as possible to the animal to achieve optimal resolution of the recorded movements and ensure that the entire ladder rung apparatus is captured in the recording.

5.3.3. Start the recording and first identify the session and the animal.

5.3.4. Place the rat on the start zone of the ladder rung apparatus.

5.3.5. The rat has to traverse the 100 cm walkway of the ladder rung apparatus three times without any interruption in order for it to qualify as a compliant run. Even if the rat reaches three compliant runs within the first three runs, a minimum of ten runs should be carried out for continuous task performance.

5.3.6. After the task, clean the apparatus and the table with 0.1% acetic acid to avoid distraction by the smell of the previously recorded rat.

5.4. Data analysis

5.4.1. Use the free video analysis software Kinovea for data analysis.

5.4.2. Select the video sequences of three compliant runs from the recording. Choose the first three compliant runs for data analysis.

5.4.3. Define the start time point and the end time point of the selected three compliant runs. The placement of the first hindlimb behind the first black line on the sidewall of the apparatus, which labels the start time point of the 100 cm walkway, defines the start time point of the run. The placement of the first frontlimb behind the second black line on the sidewall of the apparatus, which labels the end point of the 100 cm walkway, defines the end time point of the run.

5.4.4. Identify the start and end time point. Next, calculate the duration of the run across the walkway. Report data as latency time to traverse the walkway in seconds and perform statistical analysis as usual (**Figure 4C**).

5.4.5. Score the three compliant runs with the 7-category scale from Metz *et al.* by using the slow motion or the frame-by-frame function of the software (**Figure 5**)[16,17]. Determine the number of steps and the number of errors in accordance with the categories of the scale for each limb separately. The scale distinguishes between the following categories: (0) total miss (1) deep slip (2) slight slip (3) replacement (4) correction (5) partial placement, and (6) correct placement. Only the error of the initiating limb was rated. Further errors, triggered by the initial error, should not be rated.

5.4.6. Calculate the errors/step by considering the following requirements. The categories (0) total miss (1) deep slip (2) slight slip count as an error. Divide the number of errors by the number of steps for each hindlimb and each run separately. Determine the mean value of all three compliant runs for each animal and each hindlimb separately and perform statistical analysis as usual (**Figure 4D**).

REPRESENTATIVE RESULTS:

The representative results of the five minutes OFT show that the nerve crush injury five weeks post surgery has no effect on the locomotor activity (**Figure 1**).

Gait analysis with the CatWalk XT system (**Figure 2**) generates many different parameters. Selective parameters were statistically analyzed by comparing wt naïve rats with nerve-injured wt rats five weeks after the nerve crush (**Figure 2D**). Significant alterations could be detected for the run average speed, the stride length and the print area of the nerve-injured (right) hind paw. A more detailed analysis of the nerve-injured hind paw was performed with the "Interactive Footprint Measurements" module. A significant reduction of the parameters toe spread, intermediate toe spread and print length were observed in nerve-injured wt rats compared to wt naïve rats. In addition, the paw angle body axis and the paw angle movement vector significantly differ when comparing nerve-injured wt rats with wt naïve rats (**Figure 2E**).

Figure 3 presents data of motor coordination obtained through beam walking task assessment. Nerve-injured wt rats showed a significantly increased latency time to cross the beam compared to wt naïve rats five weeks post injury (**Figure 3B**). As an additional read out of the beam walking task, full slips and half slips of the nerve-injured hindlimb were counted and considered as an error for statistical analysis. The percentage of errors per step of the nerve-injured (right) hindlimb was significantly increased in nerve-injured wt rats compared to wt naïve rats.

Representative data of the ladder rung walking task (**Figure 4**) does not show significant alterations in the latency time to cross the walkway of the ladder rung apparatus (**Figure 4C**) or in the percentage of errors per step of the nerve-injured (right) hindlimb (**Figure 4D**). The analysis of the error percentage per step of the nerve-injured hindlimb considered only the score from 0 to 2 of the 7-category scale from Metz *et al*. The distribution of all score categories per step from

the 7-category scale of the nerve-injured hindlimb and the non-nerve-injured (left) hindlimb is illustrated in **Figure 4E**.

FIGURE AND TABLE LEGENDS:

Figure 1: Assessment of locomotor activity during open field test. (A) Picture of the open field test setup. Selected picture subtracted from a recorded video during open field test showing a rat in the open field arena without (B) and with (C) tracking. (D) The velocity during a five min open field test recording was investigated in wt naïve rats and wt rats five weeks after nerve crush injury. Data are shown as mean ± SEM. Statistical analysis was performed using the unpaired t test of the normally distributed data.

Figure 2: Gait analysis with the CatWalk XT system. (A) Picture of the CatWalk XT apparatus. (B) Examples of the print view showing the labeled paw prints in false color mode and examples of the timing view showing time-based gait diagram of wt naïve rats and wt rats five weeks after nerve crush injury. (C) Examples of the toe classification showing the toe spread (TS), intermediate toe spread (ITS) and print length (PL) as well as examples of the body axis view showing the body axis (white line) and the movement vector (red line) of wt naïve rats and wt rats five weeks after nerve crush injury. (D) Data of selected parameters from the "standard" classification comparing wt naïve rats and wt rats five weeks after nerve crush injury. (E) Data of selected parameters from the "Interactive Footprint Measurements module" comparing wt naïve rats and wt rats five weeks after nerve crush injury. Data are shown as mean ± SEM. Statistical analysis was performed using the unpaired t test of the normally distributed data, unpaired t test with Welch's correction of normally distributed data with unequal variance and Mann-Whitney U test of the non-normal distributed data. P-value < 0.05 was defined as statistically significant labeled as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Figure 3: Gait analysis with the beam walking task. (A) Picture and schematic drawings of the beam walking task setup. The latency time to cross the beam (B) and the percentage foot slip errors per step of the nerve-injured hindlimb during the beam walking task (C) was analyzed in wt naïve rats and wt rats five weeks after nerve crush injury. Representative picture for the start time position (D) and the end time position (E) of the beam walking task. Representative image sequence of a full slip error (F) and a half slip error (G) of the beam walking task. Data are shown as mean ± SEM. Statistical analysis was performed using the Mann-Whitney U test of the non-normal distributed data. P-value < 0.05 was defined as statistically significant labeled as *p < 0.05, **p < 0.01.

Figure 4: Gait analysis using the ladder rung walking task. Picture (A) and schematic drawings (B) of the ladder rung walking task setup. The latency time of traversing the ladder rung apparatus (C) and the percentage foot slip errors per step of the nerve-injured hindlimb during the ladder rung walking task (D) was assessed in wt naïve rats and wt rats five weeks after nerve crush injury. (E) The percentage distribution of the score category per step according to the 7-category scale from Metz *et al*. for the left and right hindlimb of wt naïve rats and wt rats five weeks after nerve crush injury. Data are shown as mean ± SEM. Statistical analysis was performed using the unpaired t test of the normally distributed data and Mann-Whitney U test of the non-normal

distributed data.

Figure 5: **Exemplary representation of each category according to the 7-category scale from Metz *et al.* during the ladder rung walking task.** Representative image sequence from the right hindlimb of category 0 – total miss, category 1 – deep slip, category 2 – slight slip, category 3 – replacement, category 4 – correction, during the ladder rung walking task. Representative pictures for category 5 – partial placement and category 6 – correct placement.

DISCUSSION:

This behavioral assessment protocol provides an overview of advantages and disadvantages as well as possible readouts of the selected behavioral test battery in a rodent model after sciatic nerve crush injury.

To obtain a comparative outcome of the sciatic nerve crush injury, a consistent crush technique is mandatory. The use of a non-serrated clamp (Ultra Fine Hemostat) instead of forceps can improve the consistency of the crush. Use the same clamp as well as the same crush position to guarantee equal nerve compression. The exclusive use of the clamp for the crush injury and handling of the clamp with care improves consistency. Also, perform the procedure of the crush injury with care. Additional damage to the nerve during surgery such as unwanted traction of the nerve can lead to undesired side effects like automutilation. Therefore, a careful nerve preparation as well as an administration of a pain reliever for a minimum of two days is recommended.

Multifactorial assessment of motor behavior can characterize the phenotype after nerve crush injury in rats at various levels. We used the OFT, CatWalk XT gait analysis, beam walking task and ladder rung walking task. A blinded experimental procedure and data analysis to experimental groups is essential for these experiments. Before behavior assessment, animals were acclimated in the testing room under testing conditions for at least 30 minutes. All the behavioral tests applied herein have the advantage that food or water deprivation are not required. 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. 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.

The assessment of gait requires a continuous task performance. The first important aspect of a continuous task performance is to convince the animals to cross the setup. To increase the motivation, place small food pellets (45 mg) at the end of the setup. In order for animals to get

familiarized with the food pellets, the pellets should be fed to them prior to testing. Also, a goal box at the end of the setup can be helpful. The setup of the CatWalk already includes a goal box, but rats sometimes hesitate to enter the goal box. Alternatively, you can add a small cage into the goal box, but the home cage from rats does not fit into the goal box. Let the rat habituate in the cage for a few minutes before acquisition. Additionally, another rat from the same home cage may be placed into the goal box or into the cage inside the goal box. Make sure that the second rat remains in the box and does not block the entrance to the goal box. 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. For the setup of the beam walking task and the ladder rung walking task, we recommend to add a goal box or the home cage at the end of the setup. To ensure consistency, the CatWalk, the beam walking task, and the ladder rung walking task should be performed at least once a week with six to ten runs.

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.

The nerve crush injury had no effect on the locomotor activity of the rat, which was measured in a five minutes OFT. Catwalk XT gait analysis is a more objective and sensitive tool to analyze gait, paw and toe placement. After an intensive training, the rats learn to cross the walkway of the CatWalk XT apparatus to the default settings. The nerve injury does not reduce the ability of the rats to cross the walkway. The automatic computation of various parameters presents the data objectively. Additional information can be gained by using the “Interactive Footprint Measurements” module and indeed, these analyzes yielded significant differences in various parameters of toe spread, print length and paw angle to body axis comparing rats with and without nerve injury.

Rats can be trained easily for the beam walking task. Differences in the latency time to cross the beam and in the number of foot slips per step of the nerve-injured hindlimb were detected by comparing naïve with crush-injured rats. A disadvantage of analyzing nerve-injured rats with the beam walking task is the size of the beam. Within the first two weeks after the sciatic nerve crush injury, the rats need assistance to cross the beam as their balance is impaired. Although some rats may be capable of crossing the beam, the risk of injuries caused by a fall is high. Nerve-crushed animals should therefore be assisted to cross the beam for the first two weeks after sciatic nerve crush injury or longer, if necessary. However, it is difficult to compare runs with and without assistance. Also, the motor balance is an important parameter assessed by the beam walking task. We considered this parameter not to be relevant to our nerve crush rat model. Therefore, scores described by Ohwatashi et al. and Johansson & Ohlsson could not be used and runs with an incomplete beam traverse were excluded for data analysis[18,19].

The 7-category scale from Metz et al. can analyze both fore- and hindlimbs and distinguish between different severity levels of errors of all limbs during the ladder rung walking task[16,17]. By analyzing the most prominent errors, which include the categories from 0 to 2, no differences of

errors per step could be detected in the hindlimb when comparing nerve-injured wt rats with naïve wt rats. Furthermore, the latency time of traversing the ladder rung apparatus did not differ between nerve-injured wt rats and wt naïve rats. Deep learning models could improve and speed up data analysis of the ladder rung walking task through an automated approach.

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.

ACKNOWLEDGMENTS:

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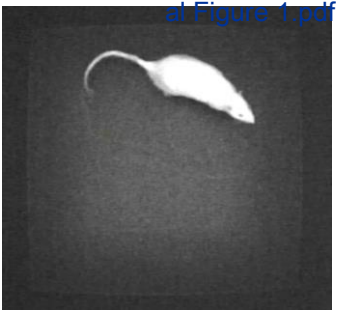
DISCLOSURES:

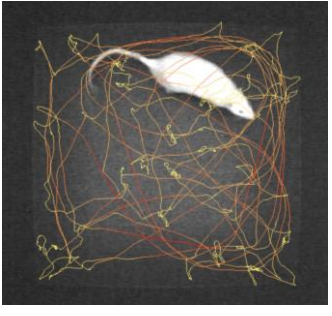
The authors have nothing to disclose.

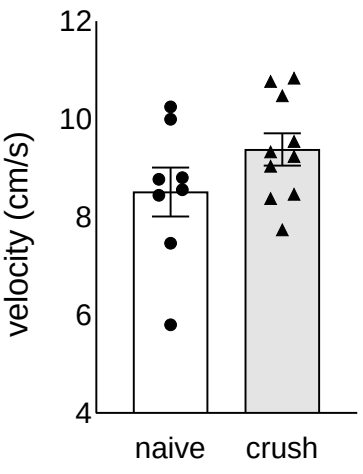
REFERENCES:

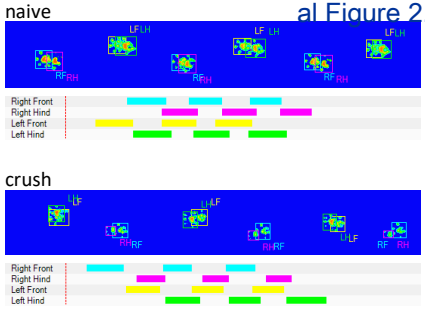
1 Iannaccone, P. M., Jacob, H. J. Rats! *Disease Models & Mechanisms.* **2** (5-6), 206-210 (2009).
2 Phifer-Rixey, M., Nachman, M. W. Insights into mammalian biology from the wild house mouse Mus musculus. *Elife.* **4** (2015).
3 Musacchio, T. *et al.* Subthalamic nucleus deep brain stimulation is neuroprotective in the A53T alpha-synuclein Parkinson's disease rat model. *Annals of Neurology.* **81** (6), 825-836 (2017).
4 Ip, C. W. *et al.* Tor1a+/- mice develop dystonia-like movements via a striatal dopaminergic dysregulation triggered by peripheral nerve injury. *Acta Neuropathologica Communications.* **4** (1), 108 (2016).
5 Rauschenberger, L. *et al.* Striatal dopaminergic dysregulation and dystonia-like movements induced by sensorimotor stress in a pharmacological mouse model of rapid-onset dystonia-parkinsonism. *Experimental Neurology.* **323** 113109 (2020).
6 Klein, A., Wessolleck, J., Papazoglou, A., Metz, G. A., Nikkhah, G. Walking pattern analysis after unilateral 6-OHDA lesion and transplantation of foetal dopaminergic progenitor cells in rats. *Behavioural Brain Research.* **199** (2), 317-325 (2009).
7 Kim, D. H., Murovic, J. A., Tiel, R., Kline, D. G. Management and outcomes in 353 surgically treated sciatic nerve lesions. *Journal of Neurosurgery.* **101** (1), 8-17 (2004).
8 Kline, D. G., Kim, D., Midha, R., Harsh, C., Tiel, R. Management and results of sciatic nerve injuries: a 24-year experience. *Journal of Neurosurgery.* **89** (1), 13-23 (1998).
9 Kaplan, H. M., Mishra, P., Kohn, J. The overwhelming use of rat models in nerve

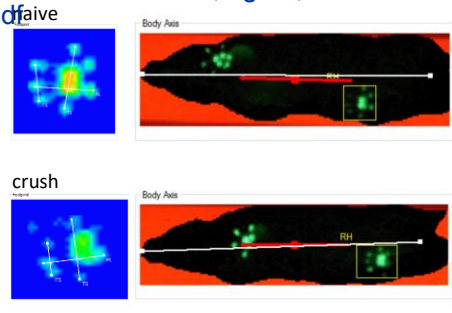
regeneration research may compromise designs of nerve guidance conduits for humans. *Journal of Materials Science: Materials in Medicine.* **26** (8), 226 (2015).
10 Bauder, A. R., Ferguson, T. A. Reproducible mouse sciatic nerve crush and subsequent assessment of regeneration by whole mount muscle analysis. *Journal of Visualized Experiments.* 10.3791/3606 (60) (2012).
11 Savastano, L. E. *et al.* Sciatic nerve injury: a simple and subtle model for investigating many aspects of nervous system damage and recovery. *Journal of Neuroscience Methods.* **227** 166-180 (2014).
12 Menorca, R. M., Fussell, T. S., Elfar, J. C. Nerve physiology: mechanisms of injury and recovery. *Hand Clinics.* **29** (3), 317-330 (2013).
13 Luis, A. L. *et al.* Neural cell transplantation effects on sciatic nerve regeneration after a standardized crush injury in the rat. *Microsurgery.* **28** (6), 458-470 (2008).
14 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. *Neurobiology of Disease.* 10.1016/j.nbd.2021.105337 105337 (2021).
15 Quartarone, A., Hallett, M. Emerging concepts in the physiological basis of dystonia. *Movement Disorders.* **28** (7), 958-967 (2013).
16 Metz, G. A., Whishaw, I. Q. The ladder rung walking task: a scoring system and its practical application. *Journal of Visualized Experiments.* 10.3791/1204 (28) (2009).
17 Metz, G. A., Whishaw, I. Q. Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: a new task to evaluate fore- and hindlimb stepping, placing, and co-ordination. *Journal of Neuroscience Methods.* **115** (2), 169-179 (2002).
18 Johansson, B. B., Ohlsson, A. L. Environment, social interaction, and physical activity as determinants of functional outcome after cerebral infarction in the rat. *Experimental Neurology.* **139** (2), 322-327 (1996).
19 Ohwatashi, A., Ikeda, S., Harada, K., Kamikawa, Y., Yoshida, A. Exercise enhanced functional recovery and expression of GDNF after photochemically induced cerebral infarction in the rat. *EXCLI Journal.* **12** 693-700 (2013).

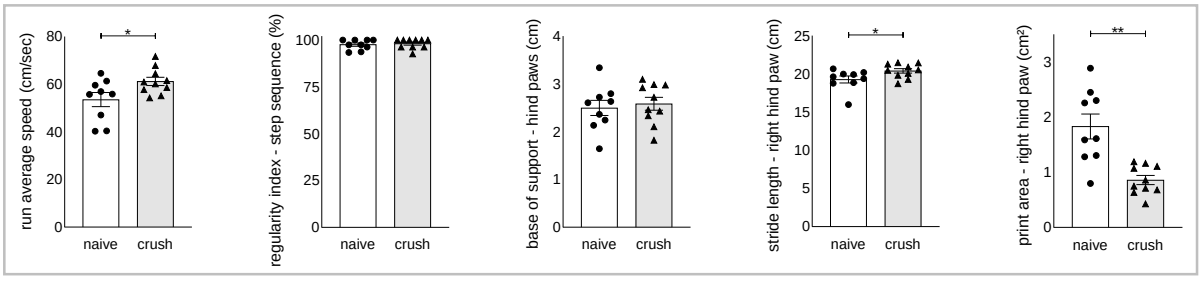


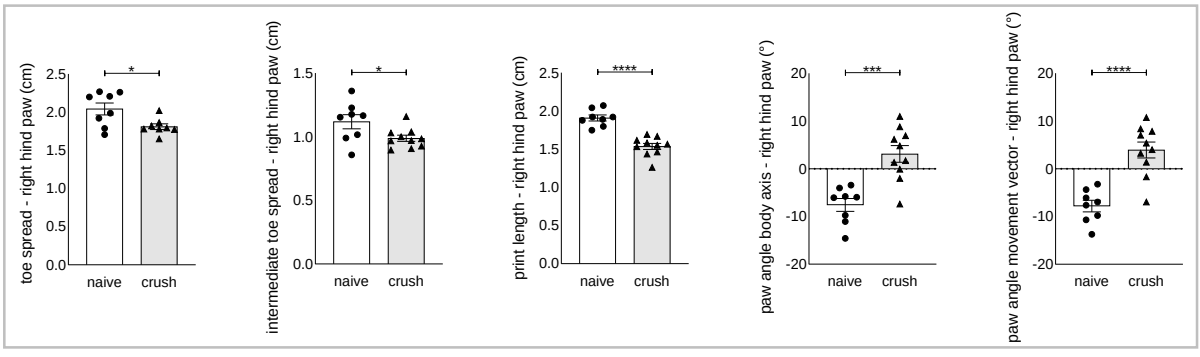












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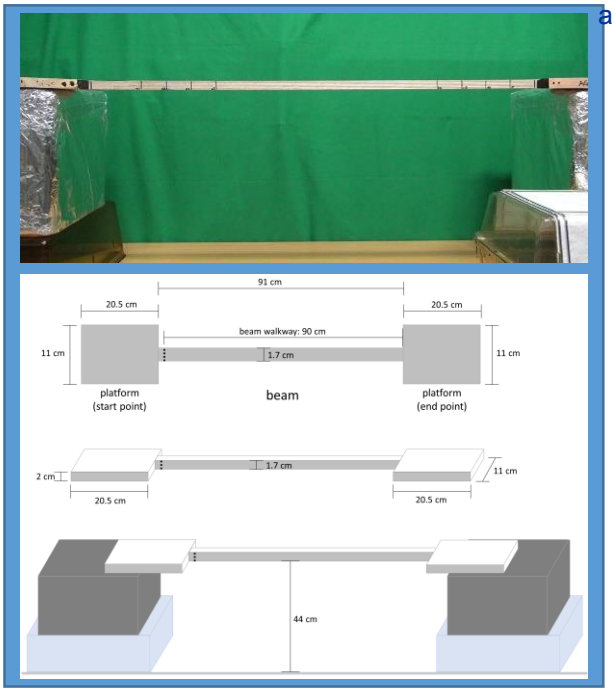

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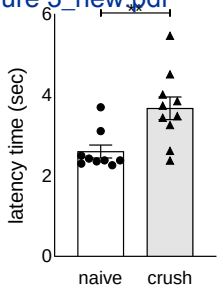

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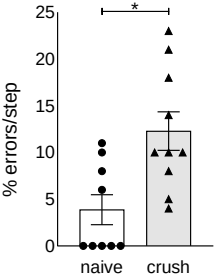

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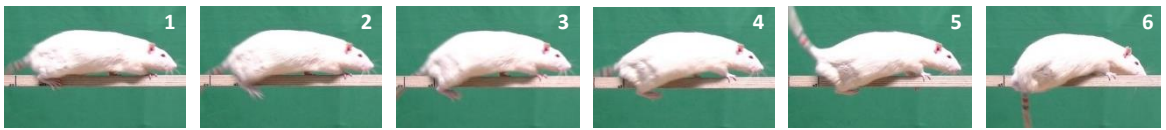

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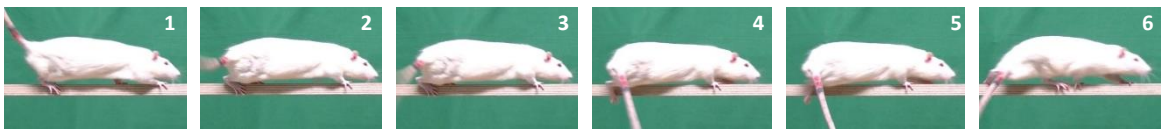

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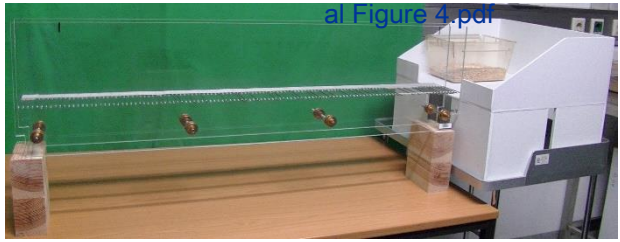

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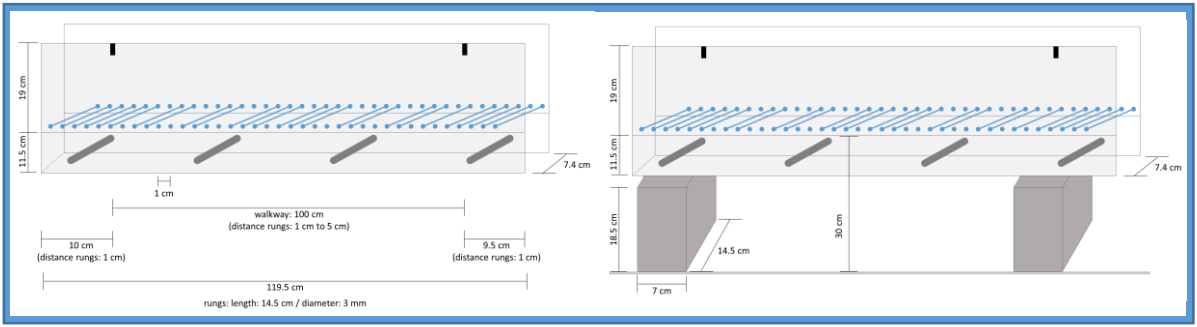

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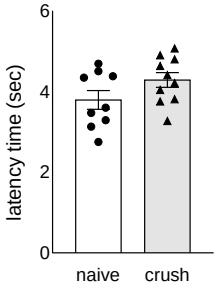

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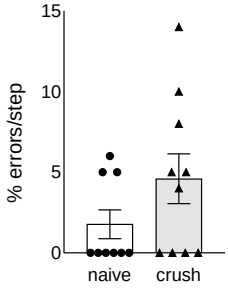

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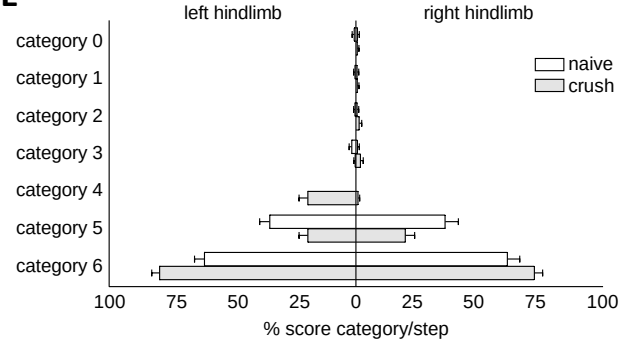
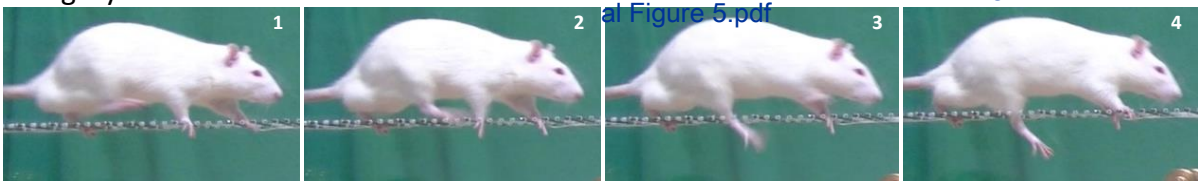
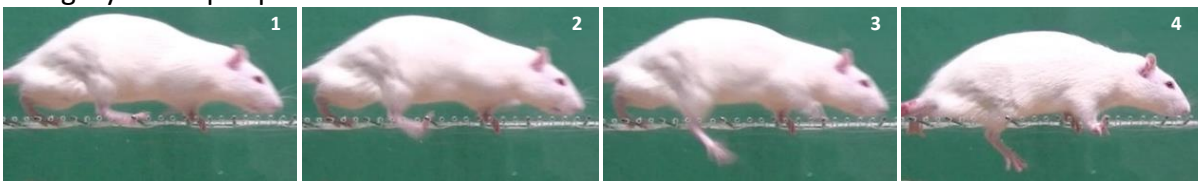


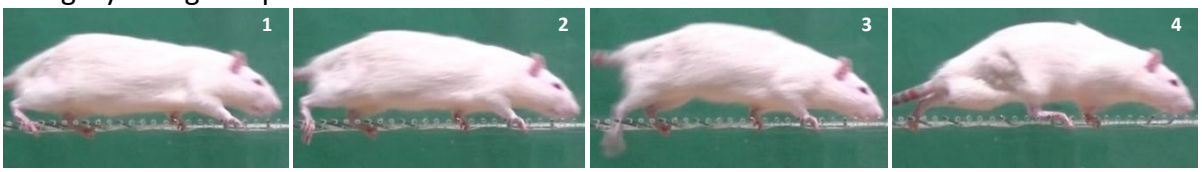
category 0: total miss



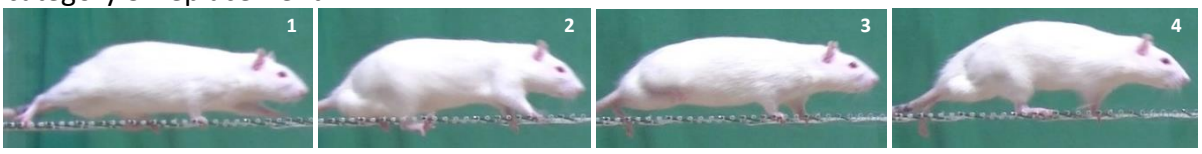
category 1: deep slip



category 2: slight slip



category 3: replacement



category 4: correction

category 5: partial placement

category 6: correct placement

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Table of Materials

Knorr et al Table_of_Materials.xlsx

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 out 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, i 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 injuy 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 preferrable 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 bram 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 auf there 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)

To Nam Nguyen, PhD
Manager of Review
JoVE

Würzburg, 4th of June 2021

Dear Dr. Nguyen,

We have included changes to the manuscript "Multifactorial assessment of motor behavior in rats after unilateral sciatic nerve crush injury" regarding the written manuscript (changes are highlighted in yellow) and the video. We also replied to the editor's comments (the editor's comments are in *italic*) as follows:

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:
1. Please renumber the protocol so that each motor behavior assessment is its own numbered step.

2. Open Field Test
3. CatWalk XT gait analysis
4. Beam walking task
5. Ladder rung walking task

We renumbered the protocol as indicated and adapted the numbers in line 281 "3.2.1. - 3.2.1.8.".

We deleted the paragraph "2. Motor behavior assessment" (line 133 – 138, verison 62606_R1) and added "A blinded experimental procedure and data analysis to experimental groups is essential for these experiments. Before behavior assessment, animals were acclimated in the testing room under testing conditions for at least 30 minutes." in the manuscript (line 630 – 632, version 62606_R1_revised).

Changes to be made by the Author(s) regarding the video:
1. Audio Editing and Pacing:
00:55 - 06:45 Audio Levels are quite high and not Balanced. Please reduce the volume so that audio level peaks average around -9 dB.

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

We hope that we could address the editorial comments in the revised manuscript and look forward to a hopefully positive response.

Best regards

Chi Wang Ip

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