A swim test for functional assessment of rodent peripheral nerve regeneration

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ARTICLE INFO

Keywords:
Peripheral nerve regeneration
Sciatic nerve injury
Swim test
Static sciatic index
Rat model
Functional assessment
Behavioral analysis

1. Background

An ideal behavioral and functional test should provide reliable, objective information on functional outcome, provide unbiased results and be easy to perform (Teixeira et al., 2020). Various techniques for behavioral functional testing and scoring such as static footprint analyses or dynamic gait assessments have been frequently applied in the field of neural regeneration research (de Medinaceli et al., 1982; Bain et al., 1989; Basso et al., 1995; Bervar, 2000; Varejão et al., 2001; Schiaveto de Souza et al., 2004; Bozkurt et al., 2008). The sciatic functional index (SFI) is one of the first quantitative parameters used to assess recovery of hindlimb motor function after sciatic nerve injury in rats and was described by de Medinaceli (de Medinaceli et al., 1982). Based on the SFI, further nerve regeneration studies followed, and subsequent modifications increased the precision of functional analyses with novel scoring methods such as the Basso, Beattie, and Beshahan locomotion scale or the static sciatic index (Bain et al., 1989; Basso et al., 1995; Bervar, 2000; Schiaveto de Souza et al., 2004). Technological advancements, including computerized video-analysis systems (e.g. CatWalk XT, Noldus Information Technology, Netherlands) enabled recording of footprints, footfalls, walking patterns and bodyweight distribution (Bozkurt et al., 2008; Cooney et al., 2016). Indeed, video imaging helped to improve these methods by enhancing reproducibility, and accuracy. These techniques, however, bring drawbacks such as the dependency on the animal’s motivation and the requirement for intensive training. Bias and errors due to the animal’s belly touching the walking track, especially when one hindlimb is not functional or full weight-bearing is not possible comprise additional limitations (Bain et al., 1989; Varejão et al., 2001; Zörner et al., 2010). Lastly, available commercial systems often are time-consuming in their use or expensive (Bozkurt et al., 2008; Bhimani et al., 2017).

Swimming as a natural behavior of rats has already received attention in the investigation of the central nervous system (CNS) in small animal models (Smith et al., 2006; Zörner et al., 2010; Xu et al., 2015).

Abbreviations: CNS, Central nervous system; GFT, Autograft group; RES, Resection group; ROM, Range of motion; SSI, Static sciatic index; ST, Swim test; SwTS, Swim toe spread index; TSR, Transection and repair group.

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https://doi.org/10.1016/j.jneumeth.2022.109663
Received 15 December 2021; Received in revised form 8 June 2022; Accepted 4 July 2022
Available online 6 July 2022
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Zörner et al. recorded videos of rodents with CNS damage (including spinal cord injury and stroke) during walking, wading, and swimming and described the applicability of several parameters for locomotion analysis (Zörner et al., 2010). Schweizer et al. were able to demonstrate the advantages of swimming motion with a simple swim test (ST) as an alternative method for assessing functional recovery in a sciatic cut and repair model after the application of systemic adipose-derived stem cells (Schweizer et al., 2020).

The swim test (ST) addresses some major drawbacks of conventional gait analysis, which makes it an interesting tool for functional assessment in experimental peripheral nerve regeneration.

In this study, we sought to evaluate a refined ST setup to monitor peripheral nerve regeneration by assessing functional return in a sciatic cut and repair model in rats.

2. Methods

2.1. Groups and experimental protocol

Animal experiments were approved by the local animal experimentation committee (Zurich, Switzerland; permission ZH 181/2018) in accordance with the Swiss animal welfare ordinance.

Eighteen male Lewis rats (200–250 g, Janvier/Charles River, Germany) were included in the study. Three rats were housed in one 2,000 cm² ventilated cage with free access to water and food. Following two weeks of acclimatization in the animal facility, rats were handled and familiarized with the functional assessment setup. Baseline performance was recorded prior to surgery (without nerve lesion), and post-surgical testing sessions were performed on a weekly basis until postoperative week 16 (Fig. 1).

Animals were randomly assigned to three experimental groups (Table 1): 1. Resection of 10 mm of the sciatic nerve (n = 6, resection group, RES); 2. Transection and direct repair of the sciatic nerve (n = 6, repair group, TSR); 3. Resection of 10 mm of the sciatic nerve and repair with a reversed autologous nerve graft (n = 6, autograft group, GFT).

2.2. Surgical procedure

Anesthesia was induced by placing the animal into an induction chamber with 4–5 % isoflurane, followed by maintenance with 2–3 % isoflurane (100 % oxygen at a flow rate of 0.3 L/min) through a snout mask. The hindlimb was shaved under general anesthesia and disinfected three times using chlorhexidine 2 % (B. Braun Medical AG, Switzerland). A skin incision was made, and with blunt dissection, careful exposure of the sciatic nerve was obtained using a dorsal approach through the thigh musculature.

| Group | n | Intervention                      |
|-------|---|----------------------------------|
| RES   | 6 | Sciatic nerve resection (10 mm)   |
| TSR   | 6 | Sciatic transection and direct repair |
| GFT   | 6 | Sciatic transection and repair with reversed autograft (10 mm) |

A 10 mm segment was resected from the sciatic nerve maintaining a 5 mm nerve stump before the bifurcation into the tibial and common peroneal nerves in the RES group, thus preventing regeneration. The TSR group rats underwent sharp transection of the sciatic nerve at the same level as the RES group’s proximal cut, followed by a microsurgical epineural repair with 9–0 nylon sutures (Ethicon LLC, USA). In the GFT group, a 10 mm long nerve graft, harvested 5 mm proximal to the sciatic nerve’s bifurcation, was sutured back in a reversed fashion with three to four epineural sutures on both ends. All microsurgical procedures were performed by the senior author under magnification with an operating microscope (Zeiss, Germany). Wounds were irrigated with droplets of 1 % Lidocaine for local analgesia. Skin closure was achieved with running sutures using a 4–0 resorbable braided suture material (Vicryl Rapid, Ethicon LLC, USA). Wounds were dressed with Opsite® Spray Dressing (Smith&Nephew, UK).

Postoperatively, the animals were returned to their cages, placed on warming plates and monitored for recovery. Postoperative pain control was ensured by administration of subcutaneous injection of buprenorphine (Temgesic®; 0.1 mg/kg) with 1 ml Ringer’s lactate for hydration. In addition, analgesics were provided in drinking water for at least 72 h (paracetamol; Dafalgan Sirup®; 200 mg/ml).

2.3. Swim test setup

The experimental setup for the ST (Fig. 2) consisted of a 150 cm long, 15 cm wide and 46 cm high glass basin filled with warm tap water (at 20–24 °C) to a depth of 24 cm. A metallic grid was fixed at one end to exit the basin into a box filled with towels. A mirror was positioned on the bottom of the pool at a 45° angle. Two additional mirrors were mounted behind the basin at a 90° angle. By positioning the mirrors in this way, locomotion could be recorded from three different perspectives with a high-speed camera (acA1920–155uc, Basler AG, Switzerland) mounted in the front. The camera was adjusted to the height of the water level and placed 160 cm in front of the tank to record a region of interest of 80 cm length in the midsection of the basin. In this region, the animal was expected to execute constant locomotion due to

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Fig. 1. Experimental protocol illustrated on a timeline. Baseline values were captured in trained animals prior to surgery. Functional assessments were performed on a weekly basis until postoperative week 16.
exclusion of acceleration in the first section and slowing down at the end before leaving the aquarium through the metallic grid. The bottom mirror enabled tracking locomotion from below during swimming. The animal’s right side was exposed to the front and placement of the mirrors behind the tank enabled simultaneous visualization of the left body side in the mirrored image. Thus, it was possible to visualize the operated and not operated hindlimbs. A custom-made LED panel was placed in front of the basin to ensure sufficient illumination due to low exposure time of high frame rate video recording. After every session, the tank was completely emptied and thoroughly cleaned.

2.4. Animal handling, training and swim testing

Two weeks prior to surgery, the rats were prepared for functional assessment: All animals were first moved into the empty glass basin to acclimate to the new environment. Then, the animals were gently placed in the water-filled basin for familiarization with water close to the ladder at the exit of the basin, later they had to swim from an increasing distance to the platform and enter the box behind through the ladder. Succeeding this task the animals were handled for the testing situation and had to swim through the entire basin. Preoperative training sessions took place until each rat could perform three uninterrupted straight swims through the 150 cm long lane. Then the baseline values were recorded. Postoperatively, the number of videos per rat was depended on its performance on each time point. If a rat performed three straight, continuous swims with a low number of forelimb strokes a minimum of 5 videos were captured. In rodents which were not able to perform an uninterrupted swim a maximum of 10 swims were recorded. We actually had no animal needing 10 runs. Motivation was encouraged by food rewards placed on a platform at the end of the pool. Food restrictions or dieting was not necessary.

2.5. Data acquisition and analysis

Basler Video Recording software (Basler AG, Switzerland) was used for capturing the swims at a rate of 200 frames per second. A series of parameters were analysed offline using the ImageJ-based open-source software Fiji (ImageJ, NIH, USA; Schindelin et al., 2012). FFmpeg, an additional Fiji plugin, was installed to enable video formats. For each animal, three videos were selected representing the best swimming performances of the testing session. Locomotion analysis was investigated on a 60 cm track in the previous adjusted 80 cm recorded at the midsection. Readouts were transferred to Excel for later analysis (Microsoft, USA).

The following parameters were used to assess functional recovery (Figs. 3, 4): mean “speed” out of the three best-performed swims was measured in cm/s. The number of “forelimb strokes” was scored manually in the three selected videos of each animal/session. Length and angulation parameters were measured at three positions in the 60 cm section. The maximum length of one stroke (“horizontal excursion”) was determined by the difference between the distance from the tip of the nose and the most posterior toe position minus the distance between the tip of the nose and the foremost toe position on a horizontal plane. Range of motion (“ROM”) of the ankle joint was calculated by measuring the minimal (“min angle”) (= flexion) and the maximum angle (“max angle”) (= extension) during swimming. The angle of the body axis drifting away from the midline displayed in the bottom mirror was defined as “trunk instability”.

Based on measurements and mathematical formula of the static sciatic index (SSI), we defined a “swim toe spread index” (SwTS) (Bervar, 2000; Smit et al., 2004). The maximum toe spread distance between the first and fifth toes and the distance between the second and fourth toes of both paws were measured during swimming and applied to the
adopted formula of the SSI (details see next paragraph).

2.6. Static sciatic index (SSI)

Animals were placed in an empty transparent plastic box (cage box) while images were captured with a perpendicular positioned camera from below in a constant distance from the box’s floor (Fig. 4). Images were captured when both hind paws were placed on the floor for later offline analysis with Fiji (NIH, USA; Schindelin et al., 2012). The distance between the first and fifth toe (= toe spread; TS) and the distance between the second and fourth toe (= intermediary toe spread; ITS) of the operated (O) and the not operated (N) side were measured using Fiji image processing software.

The toe spread factor (TSF) and intermediate toe spread factor (ITF) were calculated as follows: TSF = (OTS – NTS) / NTS and ITF = (OITS – NITS) / NITS. The SSI was calculated as proposed by Bervar (Bervar, 2000): SSI = 108.44 TSF + 31.85 ITF – 5.49.

The results were presented in percentage “%”, with 0 % showing no functional deficit and –100 % representing a complete loss of function.

2.7. Gastrocnemius muscle ratio

Muscular atrophy was assessed by determining the wet weight of the gastrocnemius muscle bilaterally after tissue sampling at the endpoint. Ratios were calculated as gastrocnemius wet weight of the operated side divided by the gastrocnemius wet weight of the contralateral side.

2.8. Histology and immunohistochemical analysis

Histology and immunohistochemistry (IHC) were carried out under the supervision of a neuropathologist (ER). At 16 weeks postoperatively, animals were euthanized and tissue was harvested for nerve specific histomorphometry. The nerve stump distal to the resection site in the RES group completely degenerated, so it could not be harvested. In the repair groups (TSR, GFT), sciatic nerves were examined from the operated and not operated side. Two 2 mm sections were excised from the regenerated nerve distal to the repair site (nylon suture material was identified under an operating microscope) and at the same level on the untreated contralateral nerve. The proximal nerve piece was fixed in 4 % glutaraldehyde in phosphate-buffered saline solution at 4 °C for 24–48 h. After post-fixation with 0.5 % osmium tetroxide, the tissue was...
dehydrated and embedded in resin. Semi-thin cross-sections were cut with a microtome and stained with toluidine blue. Immunohistochemistry was performed after formaldehyde fixation followed by paraffin embedding. The samples were washed, blocked and NF-200 and S-100 antibodies were added for overnight incubation.

Cross-sections were imaged using a Leica DM6000-B microscope (Leica Microsystems GmbH, Germany) at 40–63x magnification with immersion oil. Of each nerve cross-section, three images were taken at representative areas. Nerve fibres were manually scored in three fields of view using three different magnifications of 100 × 100 μm on the toluidine blue cross-sections. The diameter of each nerve fibre (FD) and axon (AD) was manually measured on an imagined vertical line from left to right, and top to bottom. The number of NF-200 positive axons on IHC slides were counted automatically using the command “analyze particles” in Fiji (ImageJ, NIH, USA; Schindelin et al., 2012). Myelin thickness MT was calculated as follows: (FD - AD)/2. The remyelination ratio was obtained by calculating the difference between the fibre and the axonal area, assuming circularity of the nerve fibre area to obtain the myelin area. Finally, to calculate the myelin area per mm², it is multiplicated with the nerve fibre count assumed per mm².

2.9. Statistical analysis

Prism 8.0 (GraphPad Software, USA) was used for statistical analysis. Data are presented as means ± standard deviation (SD). For better readability of the graphs, the SD error bars are presented either up or down. Statistical significance was assessed by 2-way ANOVA with Dunnett’s multiple comparisons test between the groups and timepoints for speed, recovery of horizontal excursion, ROM, maximum angle, forelimb strokes, trunk instability, SwTS, SSI. One-way ANOVA was performed to compare gastrocnemius muscle ratio, g-ratio and axon count between the groups using the Tukey’s multiple comparisons test. The unpaired t-test was used to analyze statistical difference of remyelination between the repair groups. Statistical significance was defined as: * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

3. Results

All 18 animals reached the endpoint at postoperative week 16. The effects of the procedure could be recognized postoperatively, immediately after awakening from anesthesia by functional impairment of the operated hindlimb. The study was conducted without any signs of wound healing problems, infections, or unusual weight loss. There were no signs of neuroma development throughout the whole experiment.

3.1. Functional Assessment

The ST and static sciatic index were able to detect nerve injury in the first postoperative week (baseline versus week 1); Recovery horizontal excursion all p < 0.0001; Max angle: TSR and GFT p < 0.001, RES p < 0.0001; ROM: TSR and GFT p < 0.0001, RES p < 0.01; SwTS and SSI p < 0.0001. No differences in performance could be seen between the operated side (Supp. 1. Horizontal excursion and Supp. 2. ROM). Individual trends were observed in each animal (see Supp. 1 and 2).

Horizontal excursion: similar values were seen after a significant postoperative drop (p < 0.0001) for the first two weeks. In the TSR and GFT groups, early improvement could be detected already at 3–4 weeks, whereas the autograft showed a slightly improved recovery in stroke length compared to the TSR group until week 10. In the resection group, a further worsening of horizontal excursion occurred at week 3. After steady recovery in all groups, at the last timepoint, significant (p < 0.01) recovery of horizontal excursion could be seen in the GFT (48.23 ÷ 12.32) compared to the RES (23.86 ÷ 9.49) group (Fig. 5, left).

Forelimb strokes: Forelimb action was observed in all groups in order to maintain or correct the direction and thereby compensate for impaired hindlimb function. The number of strokes increased over time, with RES animals requiring more forelimb strokes than TSR and GFT animals. (Fig. 5, right).

Ankle joint working angle: The mean of baseline working angles of healthy rats was 137.60 ÷ 1.84 degrees and dropped at postoperative week 1 ÷ 88.4 ÷ 2.94 degrees (RES), 89.07 ÷ 5.25 degrees (TSR) and 92.39 ÷ 4.91 degrees (GFT). With respect to ankle joint angulation, we could see a constant increase in the repair groups from week 10 for maximum angle. At week 16, extension in ankle joint recovered to 116.33 ÷ 19.56 degrees for TSR and 126.56 ÷ 12.34 degrees for GFT, which revealed a significant difference (p < 0.001) for GFT vs. RES (96.6 ÷ 12.34 degrees). There was an increase in ROM at week 4 in the TSR group and a steady increase in the GFT. There was minimal recovery of the ankle joint working angle in the RES group over 16 weeks with maximum angles ranging from 88.4 ÷ 2.94 degrees in week 1 ÷ 96.6 ÷ 8.15 degrees in week 16. However, there was a steady increase in ROM in the untreated RES group. (Fig. 6).

Swim toe spread index (SwTS) and static sciatic index (SSI): Results from the SSI were compared to our modified dynamic SwTS index. Both groups demonstrated a significant drop at week 1 (SSI: RES ÷ 101.14 ÷ 9.18, TSR ÷ 100.30 ÷ 5.46, GFT ÷ 98.34 ÷ 9.27; SwTS: RES ÷ 91.59 ÷ 5.02, TSR ÷ 93.33 ÷ 5.25, GFT ÷ 93.82 ÷ 3.01) and a constant recovery over time, with superior sensitivity of the SwTS in differentiating between repair and no repair groups. The SwTS did not show relevant improvement in the TSR group between week 6 (÷ 70.51 ÷ 4.74), and the endpoint at week 16 (÷ 74.25 ÷ 4.99). The SwTS index showed a constant increase in performance in the TSR group until week 8 (÷ 57.60 ÷ 17.34), and comparable development in the
GFT group until week 9 (58.69 ±/− 13.91), followed by slow progression to the endpoint TSR (54.33 ±/− 25.88), GFT (56.92 ±/− 14.91). (Fig. 7, left).

The SSI demonstrated less function in all groups as well as less consistent values. Weak recovery was observed for the SSI in the RES group until week 7 (95.12 ±/− 4.28), followed by a stepwise rise higher than the repair groups in the SSI at week 16 (RES 62.10 ±/− 20.32, TSR 77.43 ±/− 12.21, GFT 73.07 ±/− 9.21). (Fig. 7, left).

Trunk instability: Minimal drifting away from the midline was observed in all groups without statistical significance, but with slightly higher trunk instability in animals in the RES group (2.42 ±/− 0.54) when compared to the GFT (1.90 ±/− 0.51) and TSR groups (1.76 ±/− 0.52). (Supp. 3).

Speed: Velocity was slightly decreased from postop week 1, but did not differentiate between repair and no repair groups. (Supp. 4).

3.2. Gastrocnemius muscle ratio

Changes in gastrocnemius muscle weight ratio revealed significant (p < 0.0001) atrophy after resection (RES 0.24 ±/− 0.10) compared to repair groups, which did not show significance between TSR (0.54 ±/− 0.07) and GFT (0.58 ±/− 0.03) (Fig. 8a). The difference is also depicted in the gross images of the harvested muscle samples. (Fig. 8b).

3.3. Histology and immunohistochemistry

Histology and immunohistochemistry of distal sciatic nerve cross-sections confirmed axonal outgrowth and remyelination in the repair groups (Fig. 9). Manually counts of toluidine blue stained nerve fibers significantly differed between TSR (222.00 ±/− 40.39) and GFT (211.94 ±/− 43.03) per 10^4 μm2. In comparison, the automated axon counting command generated a total number of 129.18 ±/− 28.82 (GFT) NF-200 positive axons per 10^4 μm2. Myelin thickness was calculated at 0.51 ±/− 0.11 μm for the TSR group and 0.59 ±/− 0.13 μm for the GFT group. After 16 weeks, remyelination of the regenerated nerves was 33.23 ±/− 8.29 % (TSR) and 37.75 ±/− 8.83 % (GFT) compared to an untreated nerve with 71.06 ±/− 20.23 %. No significant differences were identified between naïve (0.702 ±/− 0.065), TSR (0.712 ±/− 0.060) and GFT (0.678 ±/− 0.054) g-ratios. (Fig. 10).

4. Discussion

In the present study, we devised a refined ST as an advanced motion analysis tool for generating objective and reproducible results of functional assessment in experimental peripheral nerve injury.

Functional analysis by swimming has already received considerable attention in the field of central nervous system (CNS) research, while overground gait analyses are used predominantly in peripheral nerve injury models. In our own prior studies, Schweizer et al. have shown a potential benefit of a simplified ST to investigate functional recovery after sciatic nerve injury in comparison with a gait analysis system (Schweizer et al., 2020). Zörner et al. has assessed locomotor function during walking, wading and swimming in rodents with CNS damage.
with a custom-designed setup (Zörner et al., 2010). In collaboration with the Brain Research Institute at the University and ETH Zurich, we have adopted the locomotion apparatus to study the regeneration of peripheral nerves with kinematic swimming analysis.

The application of a ST addresses some of the major drawbacks of conventional gait analyses. First, the buoyancy of water enables the animal to perform movements without having to support its own body weight, resulting in less pain and increased limb excursion (Zörner et al., 2010; Schweizer et al., 2020). This strategy leads to a reduction of bias and functional outcome can be recognized at an early stage. In contrast to other gait analysis protocols, no special dieting with food restriction was necessary (Deumens et al., 2007; Bozkurt et al., 2008). In addition, walking tests require intensive training sessions in advance of the actual procedure to ensure that the animals can perform a continuous run without interruptions. However, multiple attempts are necessary for obtaining representative prints (Hare et al., 1992; Deumens et al., 2007; Bozkurt et al., 2008; Anand et al., 2011; Walter et al., 2020). We could observe that rats quickly adapt to the ST setting, since swimming is a natural behavior (Kramer et al., 1993; Smith et al., 2004). After two preoperative training sessions, the animals were able to traverse the swim track in a continuous straight swim. Their intuitive behavior to prevent drowning and reach the exit platform at the opposite end enhances the forward momentum through the basin.

For additional motivation, a food reward was provided on the platform. In contrast to other gait analysis protocols, no special dieting with food restriction was necessary (Deumens et al., 2007; Bozkurt et al., 2008). In preliminary experiments, we observed that additional training and higher frequency of tests per session could result in rats floating around, with reduced motivation to complete runs.

Like other functional assessment tests, the ST provides a noninvasive method, which can be repeated at multiple timepoints as required by the experiment (Teixeira et al., 2020). The possibility of serial evaluations to quantify locomotion over a defined period is considered a key feature when developing a new assessment tool (Kappos et al., 2017).

Readouts from functional gait analysis, including the popular CatWalk Noldus XT apparatus, which represents a negative imprint of the rat’s feet, represent only one perspective (Bain et al., 1989; Smit et al., 2004; Pan et al., 2017). Sophisticated video-assisted walking tests highlight the disadvantage of two-dimensional and indirect imaging by positioning multiple cameras at various angles around the walking track (Nakamura et al., 2015; Tajino et al., 2018). Comparable to the mirror arrangement in our study, detailed locomotion tracking was achieved through direct visualization from three different views captured by a single camera. Accordingly, both sides (operated, non-operated) and the undersurface of the rat can be analyzed. Direct visualization was beneficial to differentiate confounders of the dysfunctional limb (Kappos et al., 2017). Other locomotion studies used joint tattooing and special landmark markings on the hindlimb to enhance measurement accuracy (Zörner et al., 2010). Landmark markings alter their relation between the joint and overlying skin due to laxity and mobile skin when swimming. We predefined separate landmarks, detailed in the methods section, for offline analyses to acquire reproducible measurements of lengths and ankle parameters.

A series of parameters identified sciatic nerve injury and functional return in the negative control group with a critical-size nerve defect and in the repair groups with direct coaptation and autografting. Parameters such as horizontal excursion and range of motion showed a steady increase in all groups, including the resection group. Findings in the resection group are related to hip and thigh motion in addition to compensatory joint movement in the paralyzed limb. In contrast, the maximum angle could be implemented as a feasible parameter, since it showed constant improvement in the repair groups, with no signs of functional return in the resection group. The minimum angle decreases from week 7–9 in the resection group, which increases the ROM in this group. The musculature in the denervated limb atrophies and the stability of the ankle joint is weakened by muscular imbalances. Passive movement during hip pushes lead to swinging of the flaccid foot in the water and result in a decrease of the minimum angle.

In addition to the trained group, healthy rats only move their hindlimbs during swimming and hold their forepaws fixed under the chest, whereas rats with impaired hindlimb function show compensatory forelimb strokes to maintain or correct direction, congruent with prior studies in CNS models (Smith et al., 2006; Zörner et al., 2010; Xu et al., 2015). No animal fully recovered from the procedure because of muscle atrophy following insufficient nerve regeneration. This explains why forelimb strokes were noted in all groups with a higher incidence in the resection group, as confirmed by gastrocnemius muscle weight ratio. Results for trunk instability were analogous, probably for the same reason.

Toe spread addresses both the peroneal and tibial branches of the sciatic nerve, which is included in several functional analyses (Bervar et al., 2000). The formula for the SFI includes toe spread distances and the paw print length (de Medinaceli et al., 1982). The SFI is one of the
most commonly used indices to assess functional recovery. In this study, recovery of toe spreading was monitored as a static parameter using the SSI. In contrast to the SFI, the SSI is more practical to perform, in addition of not being influenced by gait’s velocity and not needing extra equipment. The SSI has been shown to be a time-saving and easy technique for accurate functional assessment of peripheral nerve regeneration in rats and is calculated using static measures, without considering paw print length (Bervar et al., 2000; Smit et al., 2004).

A novel aspect of the current work was tracking toe spread from below during swim sessions, hence we called it “swim test toe spread index” (SwTS), based on the formula of the SSI, which has the advantage of gravity-free assessment and was better at depicting the differences between the groups. At endpoint, the SSI was comparable to an autograft as reported in the literature (Saller et al., 2018).

Velocity did not represent a reliable parameter of the ST in the present setup, as it could not differentiate between the repair and no repair groups. Due to a narrow speed range, dynamic speed parameters showed limitations in the characterization and differentiation of functional recovery (Kyriakou et al., 2016). In contrast, differences could be observed in a spinal cord injury model (Zörner et al., 2010). Schweizer et al. showed that animals receiving systemic stem cell therapy and nerve repair groups required less time to absolve the swim track than the group with a nerve gap (Schweizer et al., 2020).

In some of the functional parameters the GFT group showed slightly better, but not significant, regeneration than the TSR group. We interpret these results, that individual trends were observed in each animal (Supp. 1 and 2). Overall there was a high variance in standard deviation between the groups. In the TSR group, there were two rats that showed little regeneration and thus lowered the mean, whereas the standard deviation in the GFT was smaller and showed constant recovery. Furthermore, we assume there is only a small time frame where differences can be obtained. Future experiments should increase the number of tests between 2 and 8 weeks and set some mid-point biopsies in part of the animals. The relative weight of gastrocnemius muscle 16 weeks after the procedure indicated significant atrophy in the resection group compared to the repair groups (TSR, GFT). This result validated the procedure and was consistent with our functional analysis as well as previous reports (Nijhuis et al., 2013; Kappos et al., 2015). In nerve
tissue stained with toluidine blue, S-100 and NF-200, we confirmed axonal outgrowth and remyelination in the repair groups. A g-ratio between 0.6 and 0.7 indicates an optimal functional and structural nerve repair, which matched the g-ratios of our results (Chomiak et Hu, 2009). The use of S-100 positive axons as a readout enabled analysis of the slides with automatic counting software (Schindelin et al., 2012). We compared the number of manually counted axons with the result from automated software and found that manual counting was more accurate. This could be explained by the elimination of artifacts and contrast alterations before image processing. The identification of macrophage infiltration and Schwann cells with hypertrophic cytoplasm are associated with continuous remodeling as part of an immature regenerated nerve (Teymur et al., 2017; Liao et al., 2017; Stratton et al., 2018).

Additional parameters or scores have been reported for peripheral nerve regeneration using the proposed setup (Xu et al., 2015). For example, wading as an additional indicator of peripheral nerve regeneration is presumed to be superior to common walking tests through the base support provided by the buoyancy of water. Advances in automated motion analysis tools may further improve ease of performance and decrease the time for measurements and data acquisition.

5. Conclusion

The sophisticated dynamic motion analysis apparatus applied to a critical-size defect nerve model in rats using swimming as an instinctive behavior has been shown to generate reliable results for the functional assessment of peripheral nerve regeneration. Support provided by water buoyancy causes less pain for the animal and the motivation to swim encourages the rat to fully activate the injured leg during the examination, resulting in improved performance and thus less bias. The user-friendly ST procedure and setup are associated with low costs and with rapid testing sessions.

CRediT authorship contribution statement

Stefan Targosinski: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing. Anna Henzi: Conceptualization, Methodology, Investigation. Anne K. Engmann: Conceptualization, Methodology, Software, Writing. Elisabeth J. Rushing: Conceptualization, Methodology, Writing, Supervision. Andre A. Barth: Conceptualization, Methodology, Validation, Investigation. Holger J. Klein: Methodology, Investigation. Bong-Sung Kim: Methodology, Investigation. Pietro Giovanoli: Validation, Supervision.
Martin E. Schwab: Conceptualization, Methodology, Software, Validation. Jan A. Ploek: Conceptualization, Methodology, Formal analysis, Validation, Supervision. Ricardo Schweizer: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing, Supervision.

Funding

JAP was recipient of Swiss National Science Foundation funding (Grant # 31003A_169805), RS was recipient of Hartmann-Muller Foundation funding.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Irina Abakumova and Ines Kleiber for technical assistance. In addition, we appreciate S&T Microsurgical Instruments (Neuenhausen, Switzerland) for their generous support.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jneumeth.2022.109663.

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