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Original Research

Comparative Behavioral Assessment of Lewis and Nude Rats after Peripheral Nerve Injury

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Cell therapy has shown potential in the field of peripheral nerve repair, and research using rodents is a critical and essential step toward clinical development of this approach. Traditionally, most experimental peripheral nerve injuries are conducted in inbred Lewis or outbred Sprague–Dawley strains. However, transplantation of xenogeneic cells such as human-derived cells typically triggers rejection in these animals. An alternative approach is to use immunodeficient animals, such as athymic nude rats. The lack of functional T cells in these animals renders them more accommodating to foreign cells from a different host. Currently, no literature exists regarding sensorimotor behavioral assessment of nude rats after peripheral nerve injury. To this end, we compared the functional recovery during a 6-wk period of behavioral testing of Lewis and nude rats after unilateral sciatic nerve crushing injury. Three sensorimotor behavioral assessments were performed weekly: a ladder rung-walking task to assess slip ratio and cross duration, von Frey nociception testing to determine the paw withdrawal threshold thus monitoring the regaining of sensory function, and sciatic functional index evaluation to monitor the recovery of integrated motor function. Both strains demonstrated significant sensory and motor deficits in the first week after injury, with a slight regain of sensory function, reduced slip ratio, and increased sciatic functional index starting at 2 wk. No significance difference existed between nude and Lewis rats in their recovery courses. We conclude that nude rats are a suitable model for behavioral training and assessment for cell transplantation studies in peripheral nerve injury and repair.

Abbreviations: PNI, peripheral nerve injury; SFI, sciatic function index.

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An 11-y longitudinal study conducted at a Level 1 Trauma Center in Canada reported that peripheral nerve injuries (PNI) were prevalent in approximately 2.8% of trauma incidents.[27] To understand the pathology and to develop potential treatments for PNI, animal models are used. Among them, sciatic nerve injury models are the most commonly used and have provided substantial knowledge regarding the mechanisms of injury and repair.[20,22] The popularity of sciatic nerve in research is based on their large size and minimal branching, thus providing easy access for surgical manipulations.[33] In addition, the innervation of the sciatic nerve, being a mixed nerve, to the skin and muscles of the lower limbs enables functional studies, such as locomotion and nociceptive sensation analyses, to be performed.[20,22]

One potential treatment of PNI, particularly in chronic cases or those involving large nerve gaps, is the transplantation of Schwann cells.[15] Schwann cells are crucial mediators of Wallerian degeneration and axon regeneration.[8] On injury, they acutely upregulate immediate-early genes, such as c-Jun protein, and neurotrophic factors, such as nerve growth factor and ciliary neurotrophic factor.[18] In addition, Schwann cells are responsible for clearance of myelin debris through multiple pathways: myelinophagy, autophagy, and phagocytosis.[8,32] Overall, Schwann cells play a major role in facilitating axon regeneration and nerve repair. Much of the work regarding Schwann cells derives from work using rodents, but more recent studies using transcriptomic and proteomic approaches revealed differences between human and rodent Schwann cells.[25,37] Therefore, the behavior of human-derived Schwann cells should be studied in experimental preclinical injury models before being used in a clinical setting.

However, the inter-species implantation of cells typically triggers rejection in the recipient because the xenografts are immunologically incompatible.[13,19] Although immunosuppressant treatment facilitates the implantation of foreign cells, these drugs are associated with long-term risk in addition to inconvenience, because they must be injected daily, which typically leads to local inflammation.[13,29] For example, the injection of human periosteum-derived cells into a Sprague–Dawley rat model required daily intramuscular injections of FK506, which were painful to the animals.[29] To circumvent the problem, immunodeficient animals can be used. A study in which human olfactory ensheathing cells were implanted into the spinal cords of Sprague–Dawley rats without immunosuppressant revealed olfactory ensheathing cell death by 24 h, but cells implanted into athymic rats survived.[13] Athymic rats are better able to accept allogeneic cells because they harbor a spontaneous mutation in the *Foxn1* gene responsible for the development of the thymus gland, where T-cells mature and become functional.[38] Foxn1 protein is important for the development of hair, skin, and nails; consequently athymic rats lacking *Foxn1* appear to be hairless or nude. The nude rat strain provides a T-cell immunodeficient model that can retain injected human cells, thus enabling long-term observation of these cells.

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Together with histologic studies at the end of survival period, behavioral assessment is an important outcome measure and an integral component for indicating the functionality of implanted cells.[28] In addition, behavioral assessment supports longitudinal monitoring and the comparison of animals in different treatment groups. Lewis rats are a well-established strain that has been studied extensively regarding the kinetics of behavioral recovery after simple crushing injury, because of their ease of handling and decreased likelihood of exhibiting autotomy after injury.[1,11,16] In contrast, nude rats have been used in studies assessing cross-species cell transplantation after PNI, but no behavioral studies have been reported.[21] In the current study, we compared the functional recovery of Lewis and nude rats across several behavioral assays after sciatic nerve crushing injury to determine whether the responses of these 2 rat strains differ. Our findings provide insight into the use of nude rats in behavioral studies and establish a baseline for future cell implantation studies.

Materials and Methods

Animal care. All animal experiments were approved by the Animal Care Committee at the University of Calgary and adhered to CCAC standards. All applicable international, national, and institutional guidelines regarding the care and use of animals were followed.[2,9,10] A total of 6 female athymic nude rats (NIH-*Foxn1*^{rnu}) and 6 female Lewis rats (age, 2 mo) were purchased from Charles River Laboratories (Montreal, Quebec, Canada). Female rats were chosen in light of the observation that they are less likely to undergo autotomy.[36] All rats were housed in plastic static cages with filter tops and a plastic pipe for enrichment. All rats were kept in the housing facility with controlled temperature (24.5 to 25.5 °C), 15 to 20 air changes hour, and a 12:12-h light:dark cycle. The nude rats were given an irradiated diet (Pico-Vac Lab Rodent Diet 20 Irradiated, LabDiet, PMI Nutrition International, St Louis, Mo), autoclaved drinking water, and soft paper bedding to prevent soft-tissue injury and subsequent infection. Lewis rats were provided with the same caging environment except that food and tap water were not irradiated or autoclaved and that aspen wood chips were used for bedding. Animals were monitored daily throughout the experiment. Cages were changed every week for both strains. They were housed for 1 wk before baseline behavioral testing to allow time for habituation.

Surgical procedure. All 12 sciatic nerve crushing surgeries were performed by the same person to minimize interoperator variability. Rats in prone position were deeply anesthetized by using 1.5% isoflurane with oxygen, delivered through a rodent mask. Absence of corneal and withdrawal reflexes was confirmed before surgery. The surgical area was then shaved and disinfected with isopropanol, which has been our routine and a CCAC-accepted prep method for more than a decade. Buprenorphine (0.05 mg/kg SC) was given before surgery. A 2-cm incision was made on the right side of the thigh, and the sciatic nerve was exposed, with hamstring muscles retracted. The nerve was crushed at approximately 1.5 cm from the sciatic notch by using no. 3 forceps, which were fully closed on the nerve for 10 s; this process was repeated once. The nerve was then visually inspected to ensure a gap in interneural content but continuity of the epineurium at the crush site. This simple crushing injury ensures complete axotomy of all nerve fibers at the injury site but maintains interneural connective tissue continuity and a repeatable recovery course, according to extensive literature and our own experience.[1] After injury, muscles and skin were closed with 6-0 suture, and the animal was returned to its home cage for recovery on a heating pad. The analgesic meloxicam (1mg/kg SC daily) was given for 2 d postoperatively.

Ladder rung-walking task. All behavioral tests were performed by the same person. Equipment was disinfected with 70% ethanol and air-dried before introducing animals for testing. We followed a previously published crossing and scoring protocol.[23] Each session consisted of a randomized rung pattern to avoid memory effect as a confounding factor of crossing performance. The ladder was elevated and set up with a well-lit neutral start point and the home cage as the end point. The ladder path was 1m long with 20-cm-high acrylic sidewalls. The metal rungs were 3 mm in diameter and were variably placed at 1 to 3 cm along the course of the ladder. Each rat was allowed to run 5 crosses, and the best 3 satisfactory crosses were used for analysis. The 3 best crosses were determined as those having the fewest slips, shortest duration, and best gait quality. Duration (in seconds) of crossing was monitored also. Baseline readings of the hindlimb step:slip ratio and duration of ladder crossing were averaged for each strain prior to surgery. Weekly serial testing commenced 1 wk after the surgery date for as long as 6 wk; the same protocol was followed throughout the study. During early weeks after injury, some rats avoided placing their injured paws on ladder rungs; for consistency, that behavior was scored as a slip. The data were collected as video records and analyzed frame-by-frame by using Quicktime Player (Apple Computer, Cupertino, CA). The camera used was a Vixia HF R600 model (Canon, Tokyo, Japan) with recording settings of 1080 pixels at 60 frames per second.

Von Frey nociception test. A set of 20 von Frey filaments (EXACTA Precision and Performance monofilaments, Stoelting, Wood Dale, IL) was used to quantify sensorimotor recovery via paw withdrawal threshold in grams of force. We adopted the simplified up–down method for measuring mechanical nociception to perform this behavioral assessment.[5] To optimize testing duration and efficiency, the starting baseline filament was 4.31g, which is the common midrange for rats.[7] The rats were placed on an elevated metal grid that fits 6 rats at once in separate compartments and was allowed 20 min to habituate. Each filament was applied for a max of 5 times to the lateral border of the paw, which is the location of interest where the sciatic nerve innervates the paw, without collateral innervation from other nerves.[17] Prior to the surgery, 3 d of von Frey testing took place to establish a stable baseline paw withdrawal threshold record. Similar to the ladder rung-walking task, the rats were tested weekly after surgery for 6 wk.

Sciatic function index (SFI). The DigiGait (Mouse Specifics, Boston, MA) imaging system was used to assess changes in SFI before and after sciatic nerve crushing injury. The DigiGait system provided a clear ventral view of the rodents' paws and thus made the measurements conducted reliable and reproducible.[14] To calculate SFI, 3 components of the paw were taken into consideration: print length, toe spread, and intermediary toe spread.[12] Both injured and uninjured limb footprints were measured, and the equation produces a constant that ranges from 0 (normal baseline functioning) to –100 (maximal dysfunction).[12] The equation is as follows:

$$\mathrm{SFI} = -38.3 \times \frac{\mathrm{EPL} - \mathrm{NPL}}{\mathrm{NPL}} + 109.5 \times \frac{\mathrm{ETS} - \mathrm{NTS}}{\mathrm{NTS}} + 13.3 \times \frac{\mathrm{EITS} - \mathrm{NITS}}{\mathrm{NITS}} - 8.8,$$

where E indicates the experimental (injured limb), N the contralateral normal (control) limb, PL is print length, TS is toe

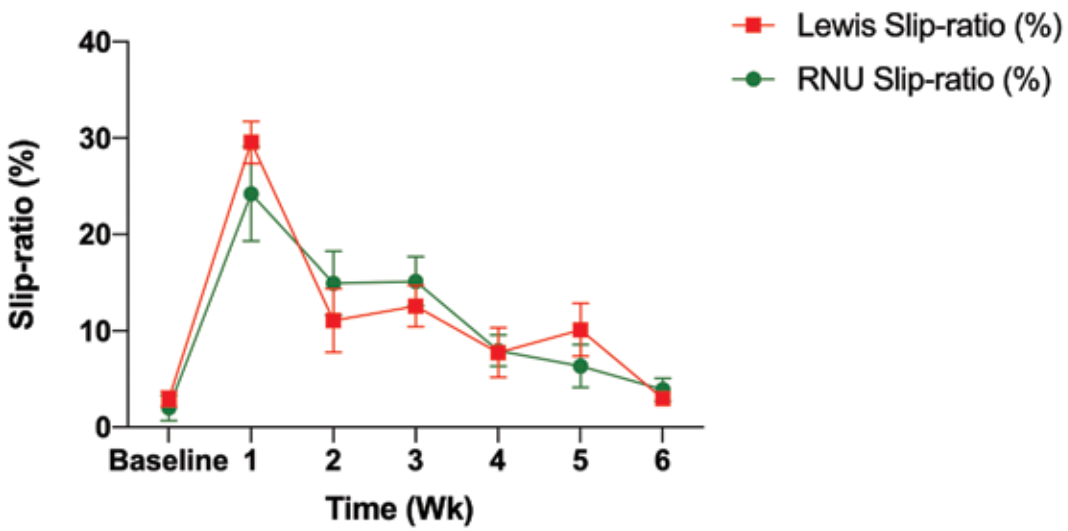

Figure 1. Step:slip ratio percentage (mean ± SEM) of Lewis and nude rat (RNU) strains in ladder tests before and after sciatic nerve crushing injury. Both strains show similar trends in recovery after injury.

spread, and ITS is intermediary toe spread.[14] We conducted this evaluation once for all rats prior to surgery and then weekly after surgery for a maximum of 6 wk. The pawprint parameters were then measured by using the measuring tool in ImageJ (NIH, Bethesda, MD).

Statistical testing. Results are expressed as means ± SEM. Data analysis was performed by using the Kruskal–Wallis test with the Dunn multiple-comparisons posthoc test to compare the 2 groups. Prism 8 (GraphPad Software, Cary, NC) was used to evaluate the differences between multiple group means. A *P* value less than 0.05 was considered significant.

Results

General health of rats. The rats maintained consistent weight gain over the study period. The mean weight of the nude rats ranged from 182 ± 6 g at the beginning of the study to 211 ± 6 g at the end of the study. The mean weight of the Lewis rats ranged from 196 ± 6 g to 221 ± 4 g. Skin infection was observed on the back of a single nude rat prior to experimental injury, and it resolved via 3-d treatment with topical polysporin. One nude rat experienced cage mate dominance in which a significant difference in weight was observed between the cage mates; the subordinate rat died during baseline recordings, resulting in 5 nude rats receiving surgery. Another nude rat died at 6 wk after surgery, possibly due to cage mate aggression, resulting in 4 nude rats for the last weekly assessment. In one case, a nude rat engaged in self-mutilation and bit off one toe after surgery. Otherwise, the health status of the remaining rodents was unremarkable.

Ladder rung-walking task. No significant difference ($P > 0.99$) was found between strains in the step:slip ratio percentage during baseline recording (Figure 1). The maximal step:slip ratio percentage was observed at 1 wk after injury. The mean step:slip ratio for nude rats was 25% ± 4%, compared with 30% ± 2% for Lewis rats. After 1 wk, the percentage decreased gradually, and both strains returned to baseline function by 6 wk (Figure 1). No significant difference between strains emerged throughout the course of testing.

The average crossing duration likewise showed no significant difference between strains within each specific week interval (Figure 2, all $P > 0.99$). The mean crossing duration ranged from 5.6 ± 0.2 s to 10.7 ± 3.7 s for the nude strain and 4.9 ± 0.5 s to 8.3 ± 1.1 s for the Lewis strain for all testing sessions.

Von Frey nociception test. No significant difference between the 2 strains existed between the baseline recordings or in the comparison with the control (left) hindlimbs for each strain (Figure 3). The week 1 time point was not plotted, because the experimental limbs were not responsive in either strain. However, by 2 wk, a significant spike compared with the respective baseline emerged in the mean paw withdrawal threshold for both strains and averaged 12.0 ± 1.3 g for Lewis rats and 17.0 ± 3.8 g for the nude strain. A general decreasing trend for both strains emerged after 2 wk and both strains returned to the baseline level at 5 wk. No significant difference between the strains was seen throughout the course of testing.

SFI assessment. SFI before injury averaged –12.0 ± 3.9 for the nude strain and –12.6 ± 1.9 for the Lewis strain; these values did not differ statistically (Figure 4). Due to the rats' inability to spread their toes after injury, SFI dropped to more negative values during week 1 but then returned to baseline by week 3. Mean SFI did not differ between the 2 strains across the 7-wk time span.

Discussion

The data from 3 behavioral tests indicates that Lewis and nude rats have similar recovery kinetics after PNI. The nude rats could be trained for behavioral studies, and therefore they are a suitable choice for xenogeneic cell transplant recipients when functional assessment is required, such as in PNI studies. However, the nude rat strain is prone to dominance issues when they are housed together, which can affect the general health of the submissive cage mate; thus, these rats should be housed individually. In addition, nude rats are prone to developing skin infection, as previously reported, and should be monitored closely and treated accordingly.[2] At the time of publication, an on-going study in our lab of 20 singly housed nude rats with sciatic nerve injury found no autophagy or skin-related issues throughout the 6-wk experimental period.

We chose the behavioral tests based on their ability to assess sensorimotor functions in relation to PNI. As compared with a tapered beam test, which assesses mainly motor coordination and balance, the ladder rung-walking task encompassed an additional sensory modality by indirectly assessing fine touch, because the rats had to step on a thinner platform.[30] Rotarod and open-field tests cannot be used to measure sensorimotor

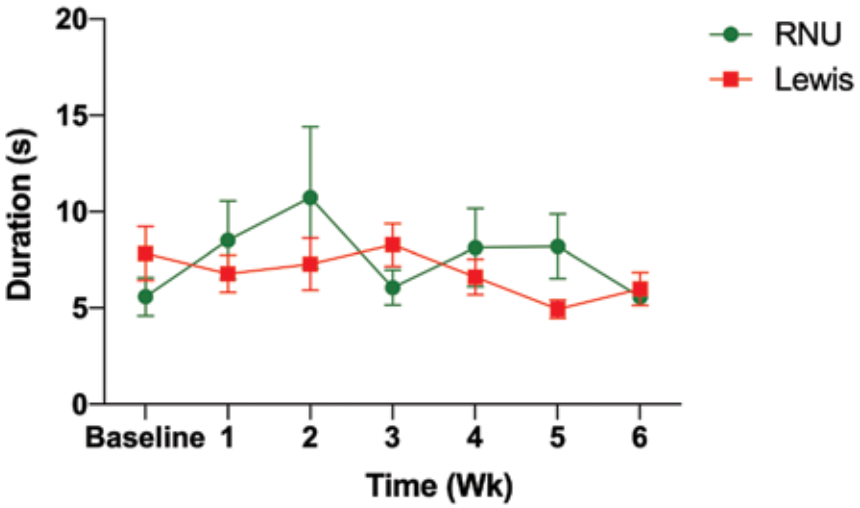

Figure 2. Cross duration (in seconds; mean ± SEM) of Lewis and RNU rat strains in ladder tests before and after sciatic nerve crush injury. No significant difference exists between the 2 strains.

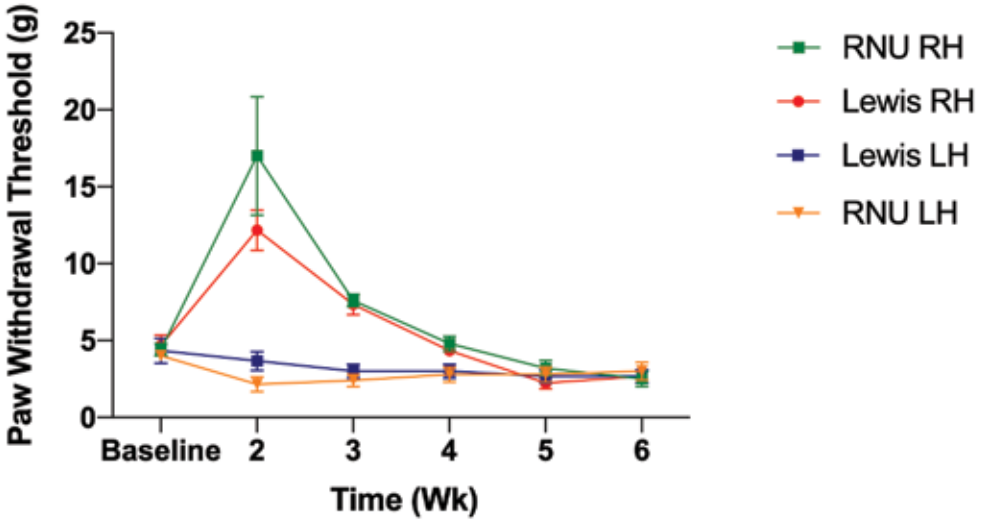

Figure 3. Paw withdrawal threshold (in grams) of Lewis and RNU rat strains before and after sciatic nerve crushing injury. Contralateral paw (left side, LH) showed consistent response throughout the course of testing. Both strains show similar trends in sensation recovery in the injured hindlimb (RH) after injury.

ability and are mainly used in studies assessing anxiety and fatigue.[30,31] The von Frey nociception test quantitatively assesses nerve regeneration by testing mechanosensitivity of the ventral aspect of the paw, which the sciatic nerve innervates. Lastly, SFI is a useful method for evaluating functional recovery after sciatic nerve injury because it assesses walking pattern and has strong interobserver reliability and reproducibility, especially with crushing (axotomic) injuries.[26,35]

According to the ladder rung-walking task results, both strains returned to baseline function within a 6-wk time frame. Although previous literature[1] had slightly different analysis parameters regarding slips, the Lewis rats showed full recovery toward baseline at 4 wk, which is consistent with the trends that we observed for both of our strains. In addition, no significant differences were found between strains in the previous study,[1] thus further supporting our results. The crossing duration for the 2 strains did not differ significantly, perhaps because we used the best 3 of the 5 trials for each animal for analysis. They represented the fewest number of slips, such that the rats could finish the task faster than they could during trials with more slips.

In terms of sensation recovery, the von Frey nociception test showed similar trends for both strains. These findings agree with those of a previous study, which showed that rats recovered in about 24 d (approximately 3 to 4 wk) after sciatic nerve crushing injury.[34] A previous study on the infiltration of T cells after sciatic nerve chronic constriction injury suggested that the lack of T cells in the injured site of homozygous nude rats reduced their mechanical allodynia and thermal hyperalgesia compared with heterozygous littermates.[24] However, the previous and our models are not directly comparable, because ours involves complete transection and subsequent regeneration of axons. For the purpose of the current study, we used von Frey filaments to examine the return of sensory function rather than to determine neuropathic pain.

We used a digital treadmill system to assess the sensorimotor function of the rats during recovery. From the paw placement prints, we were able to measure the 3 SFI parameters of print

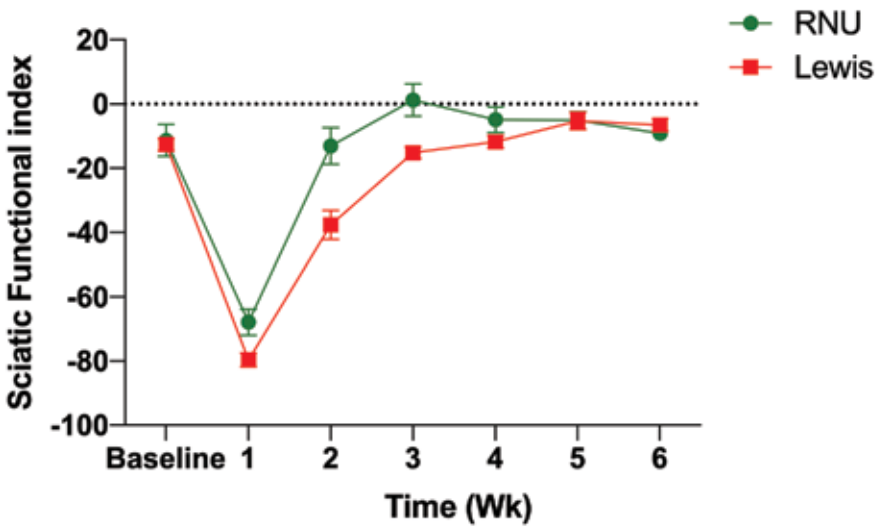

Figure 4. Sciatic functional index (SFI) of Lewis and RNU rat strains in the injured hindlimb before and after sciatic nerve crushing injury. No significant difference exists between the 2 strains.

length, toe spread, and intermediary toe spread. Figure 4 shows that both strains experienced the greatest loss of function during the first week after the induced crushing injury. However, several limitations and errors were encountered during this assessment. First, this imaging system includes software that is assumed to calculate SFI in an automated fashion. However, the software was unable to discern the color of the paw and the abdomen of nude rats due to their lack of hair; therefore, the automated paw print measurement was highly inaccurate. To overcome this technical issue, because the footage obtained from system was clear, we proceeded with manual measurement. Second, because of paw eversion or self-mutilation, some rat paws could not be measured until the second week, thus reducing the sample number and weakening the power of the analysis. Although digital automated SFI assessment has weaknesses in accuracy and repeatability due to technical limitations and errors, it nonetheless offers a proxy for functional recovery after PNI in rats.[12,14] More advanced gait analysis devices, such as CatWalk, would allow more accurate and definitive footprint analysis.[6]

The current study has a few limitations. First, the loss of animals over the course of the study reduced the statistical power. We therefore used nonparametric statistical testing, which is less powerful than parametric testing for detecting differences between outcome parameters in the 2 strains. Second, to accommodate the immunocompromise of the nude rats, we adopted a slightly different husbandry paradigm including soft bedding, irradiated food, and access to autoclaved drinking water, overlooking the possible effect of environmental enrichment on the recovery of the animals. Previous studies in spinal cord–injured nude rats showed that social and environmental enrichment significantly improved functional recovery.[4] We believe the slight changes in our housing conditions for nude rats compared with the Lewis strain likely did not significantly influence recovery. Last, due to differences in the physical appearances of nude and Lewis rats, the assessor could not be blind to strain during behavioral testing, which might have introduced biases to the analysis.

In conclusion, we found no significant differences in behavioral recovery between the nude and Lewis rat strains in the ladder rung-walking task, von Frey assessment, and SFI after sciatic nerve crushing injury. All animals returned to their respective baseline function within 4 to 6 wk. These findings support the use of nude rat strains for behavioral assessment in PNI studies.

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