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Somatosensory & Motor Research

ISSN: 0899-0220 (Print) 1369-1651 (Online) Journal homepage: https://www.tandfonline.com/loi/ismr20

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To cite this article: Roman Pavićć, Ante Tvrdeićć, Ozana Katarina Tot & Marija Heffer-Lauc (2007) Activity cage as a method to analyze functional recovery after sciatic nerve injury in mice, Somatosensory & Motor Research, 24:6, 213-219, DOI: 10.1080/08990220701745621

To link to this article: https://doi.org/10.1080/08990220701745621

Published online: 10 Jul 2009.

Somatosensory and Motor Research, December 2007; 24(4): 213–219

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Activity cage as a method to analyze functional recovery after sciatic nerve injury in mice

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(Received 1 May 2007; revised 17 August 2007; accepted 12 October 2007)

Abstract
The aim of this paper is to show the activity cage as a viable method for tracking functional nerve recovery. The activity cage measures spontaneous coordinate activity, meaning movement in either the horizontal or vertical plane, of experimental animals within a specified amount of time. This uses a minimum of researcher time conducting functional testing to determine functional recovery of the nerve. Using microsurgical forceps, a crush injury was inflicted unilaterally, on the left side, upon the 4-month-old C3H mice creating a very high degree of pressure for 6 s upon the exposed sciatic nerve. The locomotion function of the mice was evaluated using the activity cage preoperatively, 1, 7, 14, 21, and 28 days after the surgical procedure. We found that using the activity cage functional recovery occurred by 14 days after nerve crush injury. It was also shown that, coinciding with functional recovery, immunohistochemistry changes for GD1a and nNOS appeared at the level of L4, where the sciatic nerve joins the spinal column. GD1a and nNOS have both been linked to regenerative processes in mammalian nervous systems.

Keywords: *Sciatic, nerve, injury, activity cage, regeneration*

Introduction

The aim of this paper is to show the activity cage as a viable method for tracking functional nerve recovery.

In studies involving the recovery of nerve damage there is usually an intricate way to determine functional recovery of the nerve. Damage to the sciatic nerve has been linked to the sciatic functional index (SFI) since its proposal in 1982 by De Medinaceli. However, when functional recovery is a secondary concern, to be used as a marker for a different type of study, as biochemical or immunohistochemical of the nerve, the intricacies pose more of a drawback than an instrument of information. The activity cage apparatus (Ugo Basile Biological Research Apparatus, Comerio, Italy) measures spontaneous horizontal and vertical movement. Using this apparatus a minimum of researcher time is spent determining functional recovery of the nerve by conducting functional tests. Using microsurgical forceps a crush injury was inflicted upon the mice creating a very high degree of pressure for 6 s upon the exposed sciatic nerve. The activity cage was used to monitor functional recovery. Immunohistochemistry (IHC) regeneration markers GD1a and nNOS were also analyzed to evaluate on the vertebral level possible changes where the sciatic nerve joins the spinal column.

Materials and methods

Animals

Sixty 4-month-old male C3H mice weighing 30–40 g were kept in a temperature-controlled room (24°C) with 12 h light/dark cycle (lights on at 8 a.m.) with free access to water and food. Experiments were performed between 4 and 7 p.m. All experiments

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ISSN 0899–0220 print/ISSN 1369–1651 online
DOI: 10.1080/08990220701745621

were carried out according to the Ethical Committee guidelines, Medical School at the J. J. Strossmayer University in Osijek, Croatia and in accordance with Croatian law regarding the handling and treatment of laboratory animals.

Activity cage

The activity cage apparatus consists of a clear plastic box 41 cm square with 33 cm high walls (Ugo Basile Biological Research Apparatus). Every movement of an animal in any horizontal or vertical direction is recorded by 16 emitters and 16 acceptors located facing each other on either side of the cage. After the preset time elapses the printer records the number of movements in the two categories, horizontal and vertical, captured by the infrared sensor array. The animals were individually tracked for 5 min in each session. This time was randomly chosen based on operator time consumption. Every session was held under similar conditions although the order the animals were tested was random. This testing was performed preoperatively, and 1st, 7th, 14th, 21st, and 28th day postoperatively.

Surgical procedure

Using the anesthetic Isofluran (Foran, Abbott, Queensborough, UK) with a concentration from 0.75 to 1.5% volume and intraperitoneal injected solution of Ketanest (Pfizer, Vienna, Austria) diluted 10× with 0.9% NaCl, 27 mice were inducted and kept in general anesthesia. After the animal was anesthetized, the field for operation was shaved and washed with a factory prepared solution Plivasept (5 g chlorhexidine gluconate in 70% propyl alcohol; Pliva, Zagreb, Croatia). An incision was made in the medial part of the left thigh. Then, with a blunt instrument, the muscles were moved without lesion to reveal the sciatic nerve. A lesion was made on the left sciatic nerve 1 cm above the bifurcation into the tibial and peroneal nerves delivered with Martin's microsurgical forceps creating a pressure force of 49.2 N, equivalent to 1.98×10^8 Pa or 1950 times higher than standard atmospheric pressure at sea level. This pressure was held for 6 s. A non-absorbable marking suture was placed around the damaged sciatic nerve (Figure 1). The operative wound was then closed with an absorbable suture. A parallel study of 27 sham operated mice was also conducted, identical to the crush inflicted group but with only exposure of the sciatic nerve before closure. This exposed portion of nerve was also marked with a non-absorbable suture (Figure 1).

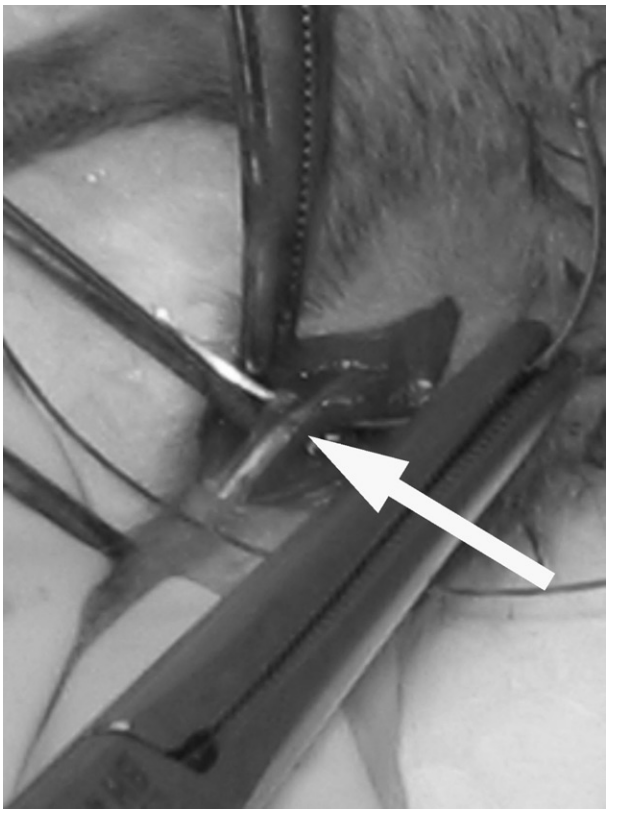

Figure 1. After crush injury was inflicted, placement of a non-absorbable suture marking crush damage (arrow) to the sciatic nerve is put into position. This consisted of a loop which did not constrict the nerve, but allowed visual orientation in the retrieval of the damaged section of the sciatic nerve during dissection.

Immunohistochemical technique

After the baseline activity cage was completed, 3 animals were killed in deep anesthesia for IHC control slices. Following surgical treatment, after each activity cage testing (1, 7, 14, and 21 days after surgery) 6 animals, 3 injured and 3 sham, per test day were killed for IHC testing. After the animal was anesthetized, the heart was perfused with Dulbecco's phosphate buffered saline (PBS buffer) followed by perfusion of 4% paraformaldehyde in PBS buffer. After this preparation the animal was dissected and the spinal cord placed in the fixative for 24 h. The samples were cryogenically prepared in PBS buffer solution containing 10% (w/v) sucrose at room temperature which was snap frozen with 2-methyl-butane at −80°C. The frozen isolated segment of spinal cord (L3–L6) was sliced to a thickness of 35 μm. The IHC analysis was done on this prepared free-floating tissue held in Tris buffered saline (TBS) (50 mm Tris/HCl, 1.5% (w/v) NaCl, pH 7.4).

The entire IHC method was performed at 4°C, and all incubations were on a shake table. First the

specimens were blocked with nonspecific blocker; 1% BSA (bovine serum albumin) and 5% goat serum in 1.5 TBS for 2 h. Blocking was with 1% Triton for nNOS and without 1% Triton for GD1a. Slices were incubated in primary antibodies diluted in blocker overnight. The primary antibody for ganglioside GD1a was used at a concentration of 0.25 μg/ml. The primary antibody for nNOS was used at a concentration of 0.5 μg/ml. Rinsing of nonspecific binding of primary antibodies with TBS was done 3 times lasting 10 min each. The secondary antibody was incubated over 4 h; in the case of anti-GD1a we used anti-mouse IgG biotin conjugated secondary and in the case of nNOS we used goat anti-rabbit IgG biotin conjugate diluted 1:1000. Rinsing non-specific bound secondary antibodies was performed the same as for the primary antibody. Bound secondary antibody is detected with the tertiary complex Vectostain ABC for alkaline phosphatase (Vector Laboratories, Burlingame, CA, USA). The incubation process lasted 2 h. Rinsing was again done in TBS 3 times for 10 min. The samples were then developed with the two-component substrate for alkaline phosphate—BCIP/NBT (Vector Laboratories) for 20 min. The slices were kept in distilled water until mounted on silanized glass and dried.

All reagents used for IHC method buffers were analytically clean (Sigma-Aldrich, Steinheim, Germany). Primary antibody for ganglioside GD1a was a gift from Dr Ronald Schnaar (Johns Hopkins School of Medicine, Baltimore, MD, USA). Antibody nNOS (neuronal Nitric Oxide Synthase) was a gift from Dr Ivica Grković (University of Melbourne, Australia). Biotinylated secondary antibody (anti-mouse IgG) was purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA), and anti-rabbit antibody was purchased from Vector Laboratories. The tertiary complex used was Vectastain ABC kit and BCIP/NBT Alkaline Phosphatase Substrate kit IV both produced by Vector Laboratories.

Statistical analysis

Statistical analysis consisted of the descriptive statistics: mean, median, and standard deviation as well as the nonparametric tests: Wilcoxon Signed Ranks test, Sign test, and Friedman test.

Results

The number of movements detected on the horizontal plane within 5 min is quite large compared to the number of vertical movements. Values were measured preoperatively, and on the 1st, 7th, 14th, 21st, and 28th day postoperatively. Looking at Figures 2 and 3

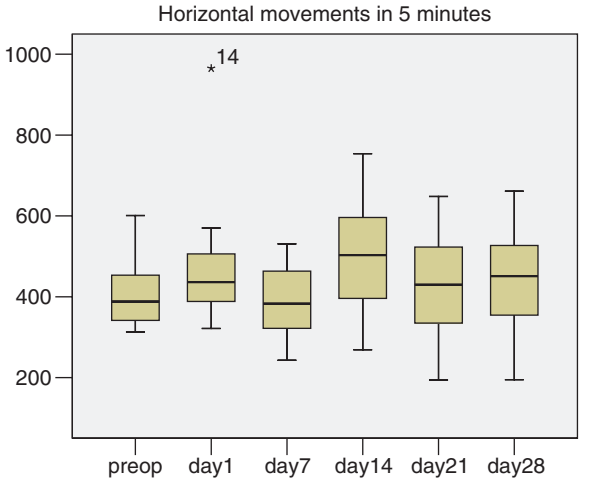

Figure 2. Horizontal movements of crush operated animals measured over 5 min. Bold line is the median value (50th percentile), box represents 25th–75th percentile, and the whiskers show minimum to maximum values, extremes are shown as ∗ (with number assigned to animal), and preop represents the preoperational values. There are no significant differences in the horizontal movements of crush operated animals. Day 1 has one extreme measurement in horizontal movement. There is not an explanation of this behavior, as this animal did not exhibit the same behavior any other day.

(horizontal movements) there are no significant results in the tested time, nor is there any statistical significance in three nonparametric tests which we tested. There is a slight drop on the 7th day postoperatively that shows muscles have ''memory loss'' which is shown the 1st day after surgery and nerve function which returns on the 14th day following surgery, but this had no statistical significance (Wilcoxon test $p = 0.363$, Sign test $p = 0.607$). The number of movements detected in the vertical direction, or rearing, within 5 min was also measured preoperatively, and on the 1st, 7th, 14th, 21st, and 28th day postoperatively. Figures 4 and 5 (vertical movements) show there was a considerable deficit in vertical activity in the operated mice. This difference is most dramatic the first postoperative day and continues until 7 days after operation. Between the postoperative 7th and 14th days recovery is noted as vertical function returns. Results in the vertical function of mice after injury to the sciatic nerve are statistically significant (Figures 4 and 5). In comparing the Wilcoxon test preoperatively and on the 7th postoperative day it is evident that there are significant statistical differences ($p = 0.013$). In the same timeframe the Sign test is statistically significant ($p = 0.013$). The surgery technique used allowed for quick wound healing (no crosscut muscle etc.) so that the sham operated animals had, as expected, no statistically significant differences in the preoperative

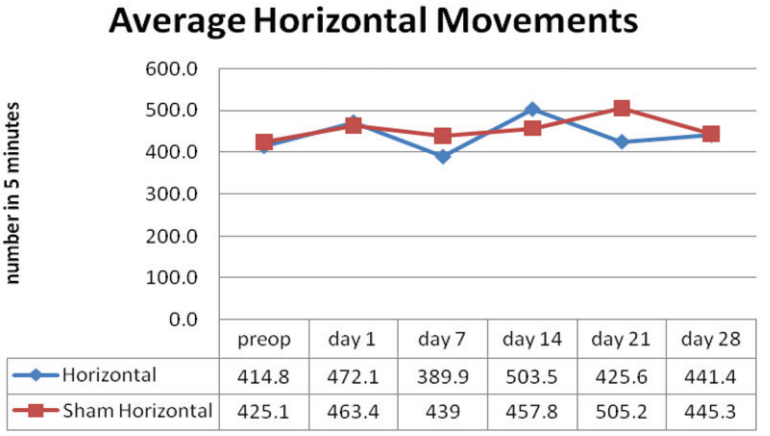

Figure 3. The horizontal movements in 5 min of the crush operated animals and sham operated animals. There are no significant differences in the horizontal movements of either group.

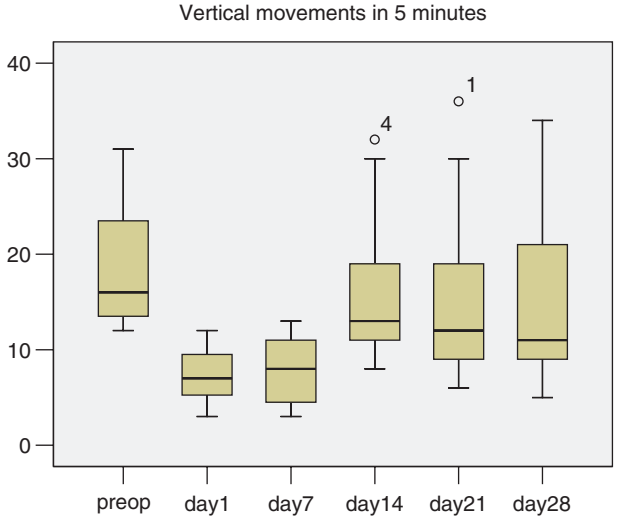

Figure 4. Vertical movements in crush operated animals measured over 5 min. Bold line is the median value (50th percentile), box represents 25th–75th percentile, and the whiskers show minimum to maximum values, outliers are shown as ○ (with number assigned to animal), and preop represents the preoperational values. There is a significant difference seen between the preop and 7th day values. There is no significance between preop and 14 days. Outliers present in day 14 and day 21 are not the same animal. There seems to be a trending to higher vertical movement, however, in the time of day 1 and day 7 when there was damage due to the crush inflicted there were no outliers.

horizontal and vertical values for sham animals compared to the 7th day and the 28th day (Figures 3 and 5).

Images of the IHC of the spinal cord at the level of L4–L5 are shown in Figure 6; 1 day (column 1), 7 days (column 2), 14 days (column 3), and 21 days (column 4) after lesion. Images in rows A, B, and E are magnified 5 times (scale is 1.0 mm), and images in rows C, D, and F are magnified 20 times (scale is 200 μm). In row A are control slices which show background staining for combined secondary antibody (α-mouse and α-rabbit), the primary antibody is omitted. Pictured in row B are IHC images with primary antibody α-GD1a. The upper quadrilateral in the first slice (1 day) indicates the magnification field of substantia gelatinosa in row C; the lower quadrilateral indicates the magnification field of ventral horn shown in row D. The arrow in row B points out increased staining in the crossover fibers 14 days after lesion. The arrow in row C shows the increase in staining in pain fibers 14 days after lesion. The arrows in row D indicate staining of ipsilateral motor neurons 7 and 14 days following lesion. Row E is the IHC slices of antibody nNOS, the quadrilateral in the 1 day slice shows the magnification field shown in row F. The arrows in row E indicate the increased staining of the substantia gelatinosa 14 days after lesion. The left side of the spinal cord is ipsilateral to the injured sciatic nerve in all images. While crush operated animals showed a dramatic change in the expression in markers GD1a and nNOS, sham animals for the same markers were continuously equal to day 1 results of crush operated animals after injury without any dynamics throughout the study period.

Discussion

There has been a great deal of research done on the effects of peripheral nerve injury upon the central nervous system. The mechanisms of nerve recovery after injury are still not fully understood. Studies are very dispersed in their subject matter, ranging from genetically altered animals to complicated learned functional behaviors. We found that a great deal of

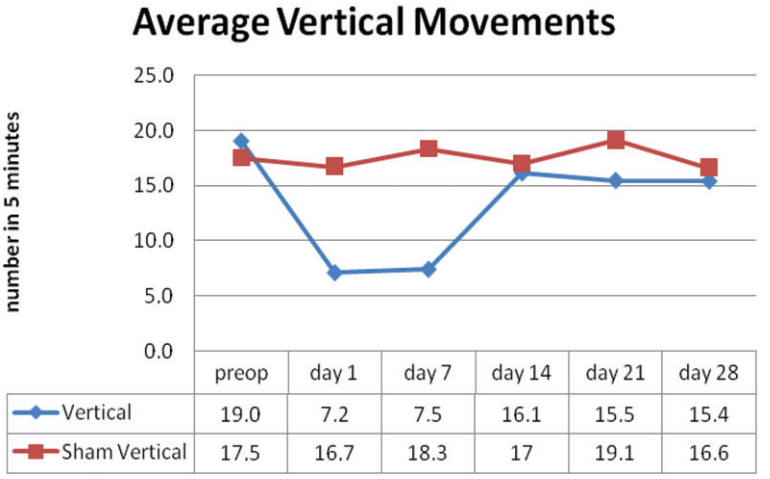

Figure 5. The vertical movements in 5 min of the crush operated animals and sham operated animals. There is no significant difference in the vertical movements of the sham operated animals. There is a significant difference between preoperative and day 7. Following nerve recovery there is no significant difference between preoperative and day 28.

Table I. The average activity in 5 min, horizontal and vertical, median, and standard deviation for injured animals.

	Horizontal movements			Vertical movements		
	Average	Median	Std dev.	Average	Median	Std dev.
Preoperative	414.8	388	88.3	19	16	7.1
1 day postoperative	472.1	436	154	7.2	7	2.8
7 days postoperative	389.9	383	88.7	7.5	8	3.7
14 days postoperative	503.5	503	148.4	16.1	13	9.1
21 days postoperative	425.6	430	142.1	15.5	12	10.4
28 days postoperative	441.4	451	139.9	15.4	12	11.7

time is spent evaluating functional recovery even when the thrust of our research was in a different area. Therefore, we attempted to find a less time-consuming way to assess sciatic nerve recovery so that with this as a guideline we could better assess when healing had occurred and therefore know when to make our other inquires. Frequently the activity cage method is used for experiments involving behavior or psychologically tested animals (Ambrogi Lorenzini et al. 1987), especially with aggression. Experiments using the activity cage method have also been done with rats in the fields of general toxicology and psychopharmacology (Joffe et al. 1990), and testing of spontaneous behavior with the testing of genetically altered mice; Pan et al. (2005) used an activity cage over two periods of 24 h to measure the hyperactivity in wild type and various genetically mutated mice but this is the first time this method has been used to track nerve regeneration after a peripheral nerve injury. Usually some type of observational method is used to track functionality; some of these methods are more subjective, so the activity cage allows for objectivity. De Souza found that the open arena exploratory activity was a poor method to detect sciatic nerve impairment (Schiaveto de Souza et al. 2004). De Souza's experiments were done with rats, and conducted over a period of 4 min. In the open arena spontaneous movement was measured with hand operated counters and stop watches to score locomotion and rearing frequency. In our experiment the vertical or rearing showed functional recovery following sciatic nerve crush injury in mice 14 days after injury. Using a similar surgical technique for the nerve crush injury and SFI calculated from walking track footprint analysis, recovery of neurologic function was shown by the 20th postoperative day (Yao et al. 1998). Our activity cage results show recovery similar to that using SFI (Pavić et al. unpublished data), however, the time factors in evaluating values are greatly increased using the manual SFI method. The activity cage is not as sensitive a method as walking track analysis, but it is acceptable as a method for showing regeneration after sciatic nerve injury and sensitive

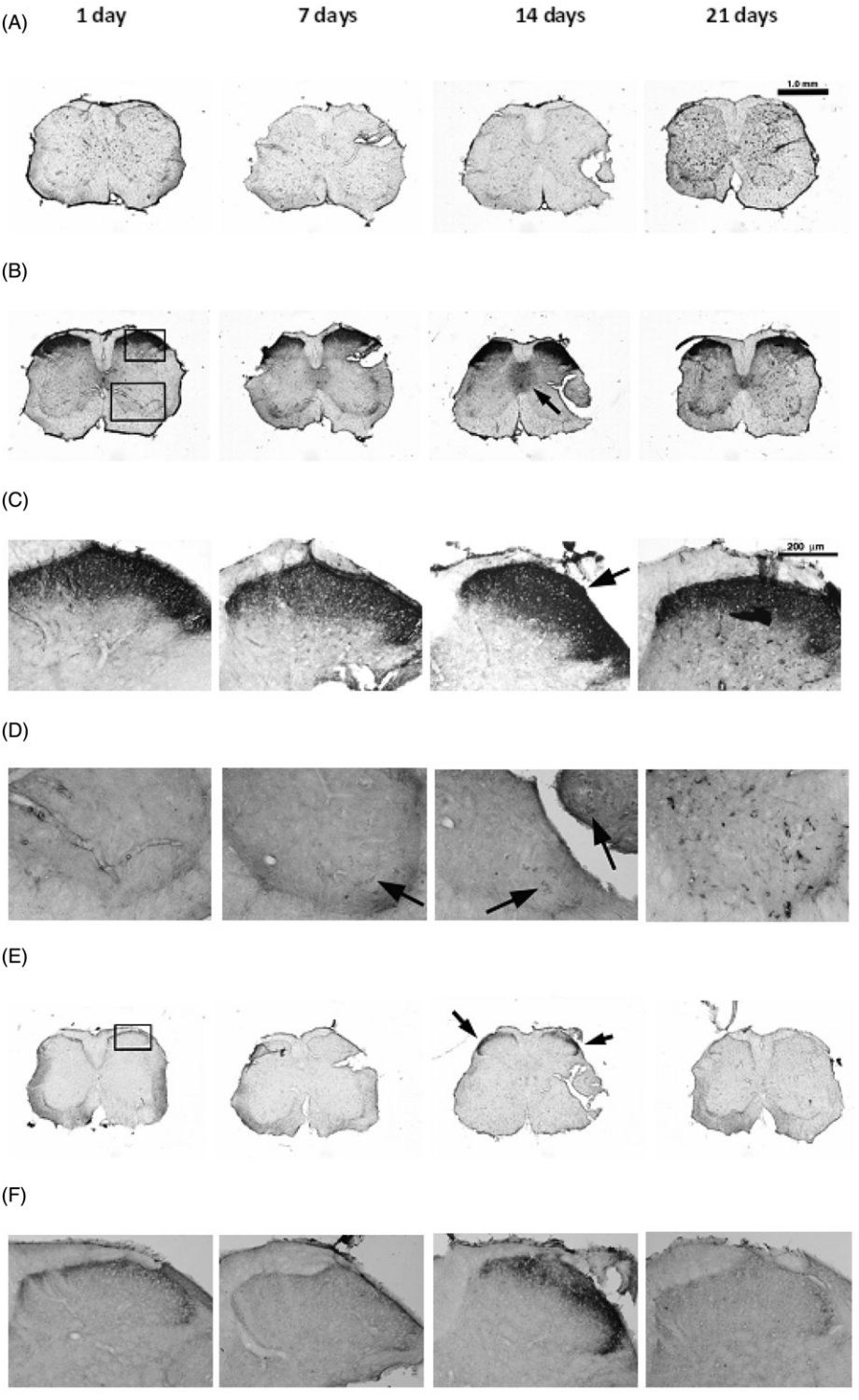

Figure 6. Immunohistochemistry slices of injured animals shown through 5× magnification (scale equals 1.0 mm) in rows A, B, and E and 20× magnification (scale equals 200 µm) in rows C, D, and F.

enough to document functional recovery 14 days after injury. Though we did not see a return to levels equal to before injury this difference was not significant (Wilcoxon $p = 0.532$, Sign $p = 1.0$). It is probable that motor function, although fully recovered with the conventional methods, was still impaired as Vogelaar et al. found, the animals did not put full weight on their previously injured paw (Vogelaar et al. 2004). It is also found in the literature that after surgery mice self-mutilate, we however did not have any cases of this in our study.

The surgically delivered crush injury is an adequate method for tracking nerve regeneration. This injury is a type of axonotmesis, loss of axonal continuity resulting in Wallerian degeneration distally on the sciatic nerve, but the myelin sheath retains its integrity. Using IHC tests on the vertebral sections at the level of L4 and L5 where the sciatic nerve joins the spinal cord, the activity in regeneration markers strongly suggests regeneration took place.

Both GD1a and nNOS have been studied for their roles in nerve regeneration in mammals. Without nNOS regeneration is extremely delayed (Keilhoff et al. 2003) and we show how this marker exists on both sides of the vertebral segments (mentioned above) after one-sided sciatic nerve injury. Antibodies to GD1a are associated with acute motor axonal neuropathy (motor/axonal variants of Guillain–Barré syndrome). Mouse antibody GD1a clearly had preferential binding to motor nerve fibers compared with sensory fibers (Gong et al. 2002). Looking at the IHC results for GD1a ganglioside compared with the control slices, we can see coloring exists in places known to contain sensory and motor axons in vertebral level L4 and L5. We also see an increase of staining in the motor neurons in the ventral horn of the spinal cord, ipsilateral and contralateral from 7 to 21 days, however, sham operated animals also have weakly stained neurons, as well as never operated animals in the lower lumbar and cervical segments so to comment on the significance of these results it would be necessary to conduct a quantification of the entire segment of the spinal cord which contacts the sciatic nerve in a future study. The same is true for the nNOS results which show an increase in the substantia gelatinosa 14 days after lesion, but IHC is not a good method for quantification.

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