Chapter 32

Evaluating Motor Neuron Death in Neonatal Rats Subjected to Sciatic Nerve Lesion

Andre Schwambach Vieira, Alexandre Cesar Santos de Rezende, and Fabio Rogerio

Abstract

Neonatal sciatic nerve lesion is a useful experimental model for the study of neuronal cell death. Sciatic nerve transection or crush is the most frequently used approach to evaluate motoneuron loss in the lumbar enlargement of the spinal cord. Here we describe and illustrate the surgical procedures performed in our laboratory to assess motoneuron cell death and the related cellular mechanisms.

Key words: Neonatal rat, Sciatic nerve, Transection, Crush, Motoneuron, Neuronal cell death

1. Introduction

During neurogenesis, programmed cell death is considered to play a role in the establishment of neural pathways. Specifically, neurons produced in excess would be excluded through cellular mechanisms dependent on the availability of trophic factors (1, 2). Since the original studies conducted by Rita Levi-Montalcini (3) on the nature and characterization of neurotrophic factors, it has become clear that distinct populations of neurons are sensitive to specific trophic molecules. Innervated organs and cells of the central and peripheral nervous systems are recognized as sources of such molecules. In addition to the evidence obtained during embryogenesis, the dependence of neuronal cells on trophic factors synthesized by their targets can be experimentally highlighted after transectioning or crushing the fibers of a peripheral nerve (1, 4–10).

A commonly used model to study the effects of the disruption of peripheral trophic support on neurons is transectioning the sciatic nerve of rodents within the first postnatal week. In this approach, the nerve is completely sectioned and separated in two stumps,

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proximal and distal, which are kept distant one from the other. Therefore, since physical contact between the stumps is not allowed, axonal regeneration is impaired (11). When performed at the first postnatal day (P0), such type of injury determines the loss of approximately 100% of the lesioned motoneurons in the lumbar enlargement of the spinal cord. Such extensive neuronal cell loss is not achieved when the lesion is performed in older animals, particularly adults. Besides disruption of trophic support from the target, it is hypothesized that immature nonneuronal cells present in the peripheral nerves, especially Schwann cells, would not be able to provide the lesioned motoneurons with trophic factors. The opposite would be true for the sciatic nerve of adult animals (5, 12–14).

Another experimental model used to evaluate motoneuron damage is sciatic nerve crush. Conversely to the nerve fiber transection model, the axons are not totally sectioned. In fact, as neural fibers are disrupted by compression, the nerve stumps are thought to remain connected, which would allow for neuronal regeneration. Specifically, cellular recovery would be favored by a microenvironment established at the injury site, in which growth factors and chemokines released by cells and molecules of the extracellular matrix would allow remodeling of the nerve stumps, axonal recovery, and neuronal regeneration, at least in part, after the lesion. However, it is important to emphasize that Schwann cells of neonatal rats have reduced competence to support regenerating motoneurons when compared to adult Schwann cells. One hypothesis that may be put forward is that immature Schwann cells have reduced production of trophic factors (1, 5, 15, 16).

Motoneuron lesion induced by sciatic nerve transection or crush in neonatal rats is an experimental model that can be used in many research fields of neuroscience, such as neuronal cell death pathways, peripheral nerve regeneration, and functional recovery and evaluation of trophic factors and neuroprotective agents. In our laboratory, we have focused on the use of exogenous growth factors and neuroprotective substances on motoneuron survival after sciatic transection in neonatal rats. In particular, we have been investigating neuronal cell survival and the possible related cellular pathways through motoneuron counting and evaluating protein and gene expression (9, 10, 17, 18). Here we describe the surgical procedures currently performed in our laboratory to induce motoneuron injury in the lumbar spinal cord of neonatal rats by either transectioning or crushing the sciatic nerve.

2. Materials

2.1. Surgical Procedure

1. Microscissors
2. Microforceps
3. Inverted microforceps

4. Microneedle holder
5. Scalpel #11
6. 8-0 suture silk
7. Glass Petri dish
8. Cotton swabs
9. 100-W incandescent lamp
10. Surgical microscope

2.2. Transcardiac Perfusion

1. Ketamine (75 mg/kg)/xylazine (15 mg/kg)
2. 1-mL plastic syringe
3. Surgical table
4. Surgical scissors
5. Forceps
6. Hemostatic forceps
7. 28-G needle attached to flexible tube
8. Peristaltic pump
9. Microscissors
10. 0.9% NaCl solution containing 400 U/L of heparin
11. 4% Buffered formaldehyde solution

2.3. Spinal Cord Dissection

1. Surgical scissors
2. Forceps
3. Scalpel #11
4. Microscissors
5. Microforceps
6. Surgical table
7. Surgical microscope

3. Methods

All the experimental procedures described herein were approved by the Committee on Animal Care of the State University of Campinas (protocol # 509-1).

3.1. Surgical Procedures

1. Separate rat pups from their mother in a small plastic box and maintain them under a 100-W incandescent lamp until the experiment is complete (see Note 1).
2. Immerse one rat in crushed ice for 4 min to induce anesthesia by hypothermia (see Note 2).

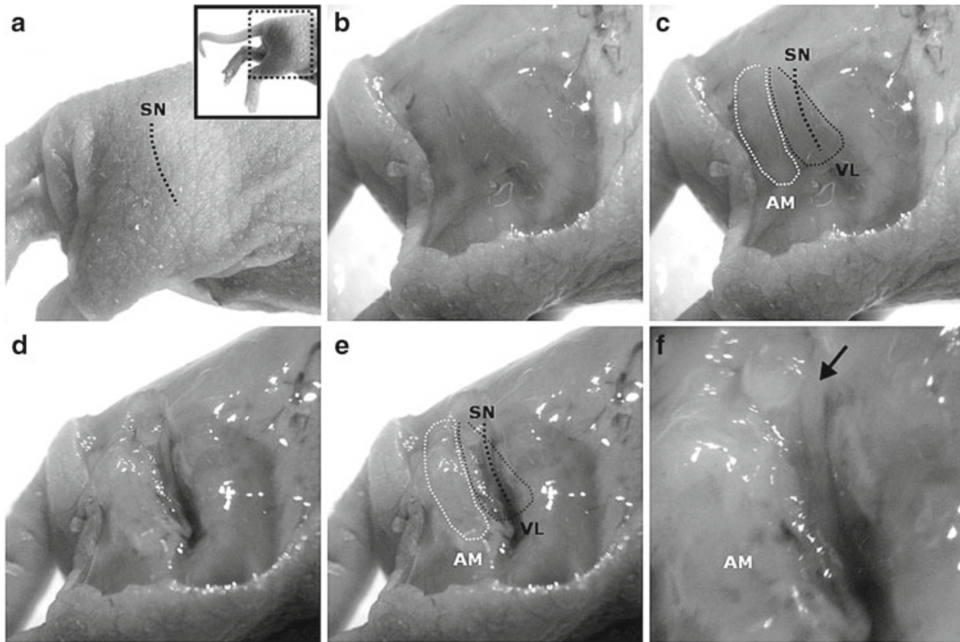

Fig. 1. Anatomical location of the neonatal rat sciatic nerve as seen under the surgical microscope. (**a**) The approximate location of the sciatic nerve (SN) is shown as a *dotted line* on the skin. The inset shows the region magnified in (**a**). (**b**, **c**) The SN runs under the *vastus lateralis* (VL) and the *adductor magnus* (AM) muscles. (**d**, **e**) The VL and AM muscles were removed to expose the SN. (**f**) Higher magnification of the SN. The *arrow* indicates the proximal region of the nerve. (**c**, **d**) The nerve and the muscles are indicated by *dotted lines.*

3. Place the anesthetized pup on a Petri dish. From this moment on, perform all procedures under the surgical microscope.
4. Make a 1-mm incision on the skin of the midthigh 2 mm posterior to the upper half of the femoral bone (see Figs. 1 and 2).
5. Introduce the microforceps between the muscles *vastus lateralis* and *adductor magnus* and separate them by repeatedly opening and closing the forceps (see Figs. 1 and 2).
6. The sciatic nerve runs under the muscles mentioned above (item 5). Using the microscissors and the microforceps, dissect the nerve until the site where it crosses the *incisura ischiadica*.
7. For sciatic nerve *transection*, hold the nerve with the microforceps and cut it using the microscissors, 3 mm proximal and distal to the region being held. Remove the nerve segment which was isolated (see Note 3; Fig. 2).
8. For sciatic nerve *crushing*, press the nerve using the inverted microforceps with fine tips. Keep such pressure for 30 s (see Note 4).
9. Reapproximate the *vastus lateralis* and *adductor magnus* muscles and suture the skin using the 8-0 silk.

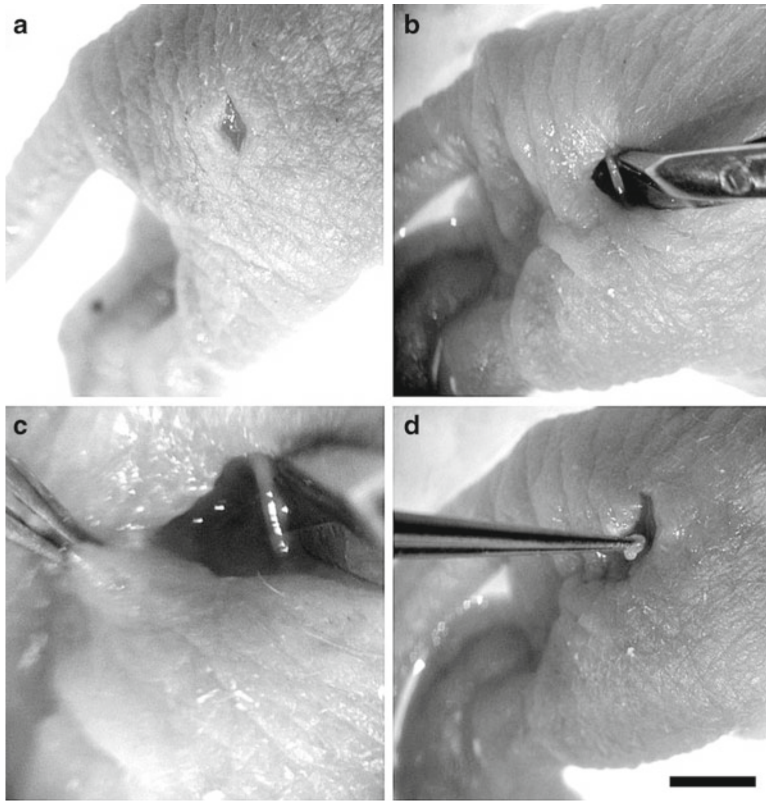

Fig. 2. Anatomical location of the neonatal rat sciatic nerve as seen under the surgical microscope. (**a**) Incision on the midthigh skin. (**b**) Exposed sciatic nerve after separation of the *vastus lateralis* and the *adductor magnus* muscles. (**c**) Higher magnification of the region shown in (**b**). (**d**) The segment of nerve removed after transection is shown between the tips of the microscissors. Bar: 3.0 mm (**a**, **b**, **d**), 1.2 mm (**c**).

10. Place the neonate rat under the 100-W incandescent lamp until recovery is observed (see Note 5).
11. Return the pups to their mother (see Note 5).

3.2. Transcardiac Perfusion

1. Separate rat pups from their mother in a small plastic box and maintain them under a 100-W incandescent lamp (see Note 1).
2. For anesthesia, administer the solution of ketamine (75 mg/kg)/xylazine (15 mg/kg) intraperitoneally.
3. When the rat does not display any pedal reflex, fixate it on the surgical table.
4. Make a skin incision through the midline, extending from the sternal angle to the pubic region.
5. In the abdominal region, make a bilateral incision parallel to the subcostal region and a midline linear incision. In this procedure, the muscular and peritoneal membrane will be sectioned, exposing the abdominal cavity.

6. Use small scissors to make an incision in the diaphragm in order to access the thoracic cavity.
7. Make bilateral sections along each lateral part of the thoracic cage. Lift the released ribs using the hemostatic forceps in order to expose the heart.
8. Gently introduce the needle in the left ventricle and cut the right atrium with microscissors (see Note 6).
9. Use a peristaltic pump to perfuse the rat with saline solution, followed by a 4% buffered formaldehyde solution (see Note 7).

3.3. Spinal Cord Dissection

1. After perfusion, fixate the rat in the prone position (*decubitus ventralis*) on a surgical table.
2. Make an extensive dorsal skin incision through the midline to expose the paravertebral musculature. From this moment on, perform all procedures under the surgical microscope.
3. Gently remove the paravertebral musculature using a scalpel and the vertebrae with the microforceps. Then, section the meningeal covering to expose the spinal cord (see Note 8).
4. For motoneuron investigation, isolate the lumbar enlargement from the remaining spinal cord using a scalpel. Remove the lumbar enlargement from the spinal canal, sectioning the nerve roots and meningeal membrane using microscissors.
5. The isolated lumbar enlargement may be processed using standard histological protocols for paraffin embedding or frozen processing (see Note 9).

4. Notes

1. We routinely use an incandescent lamp (100 W) under which the pups are kept warm throughout the experiment, as they are isolated from the mother and placed in a plastic cage. A distance of approximately 20 cm between the lamp and the rat is usually sufficient. For anesthetic recovery, the same approach is performed.
2. Deep hypothermia in young rodents is an efficient technique to induce anesthesia (19). Since the pups are not pharmacologically treated, there is no risk of death associated with overdose. After a short period of being unexposed to the incandescent lamp, the rat must be totally immersed in crushed ice until limb movements completely stop. A period of 4–5 min of hypothermia (counting from the immersion) is sufficient to elicit anesthesia for a surgical procedure of about 10 min.
3. It is crucial to keep the nerve stumps at a distance to avoid regeneration. For this goal, an additional useful procedure is to

retract the nerve endings in opposite directions using the microforceps before approximating the muscles for wound closure.

4. The inverted microforceps correspond to custom-made microforceps with a 1-mm tip that opens when pressed and closes when released, that is, the opposite to a standard microforceps. In our experience, such inverted microforceps are extremely helpful to ensure a constant and reproducible pressure applied to the sciatic nerve in crushing experiments performed in different occasions.
5. After the surgical procedure, dry the animals and place them under the incandescent lamp until indirect signals suggestive of anesthetic recovery are observed. We consider active respiratory and upper limb movements as reliable observations that the pup has recovered from anesthesia and is ready to be returned to its mother. Before returning the pups to their mother, make sure that the surgical wound is completely free of blood clots. The presence of blood may interfere with the acceptance of the mother to its pups and eventually with breastfeeding.
6. During transcardiac perfusion, take extreme care to avoid the rupture of the cardiac septum. A useful procedure is to attach a plastic catheter to the 28-G needle. The catheter should be 2 mm shorter than the needle in order to allow the introduction of only 2 mm of the needle inside the heart. The rupture of the septum may be inferred by increase in lung volume and elimination of liquids through the upper airways.
7. In our experience, the ideal perfusion rate is 3.0 mL/min. At this rate, the perfusion with saline takes approximately 5 min, and with formaldehyde, approximately 15 min. After the perfusion, we usually keep the whole rat immersed in a buffered formaldehyde solution for 12–24 h. The goal of this step is to avoid the occurrence of histological artifacts (see Note 8).
8. Extreme care should be taken when dissecting the spinal cord. Excessive manipulation of inadequately perfused specimen or after insufficient period of fixation may induce the occurrence of the so-called dark neurons. In hematoxylin- and eosin-stained sections, dark neurons are shrunken, irregular, and densely basophilic cells. This particular histological artifact may interfere with the morphological evaluation of motoneurons, mainly being confused with dying neurons (20).
9. Morphological evaluation of spinal motoneuron loss induced by sciatic lesion in neonatal rats may be performed through different approaches. Since the sciatic nerve is constituted of motoneurons whose bodies are in the ventrolateral region of the lumbar enlargement (L4 and L5 segments mainly), a peripheral insult allows one to carry out evaluation by counting the remaining cellular bodies. A possible semiquantitative evaluation consists

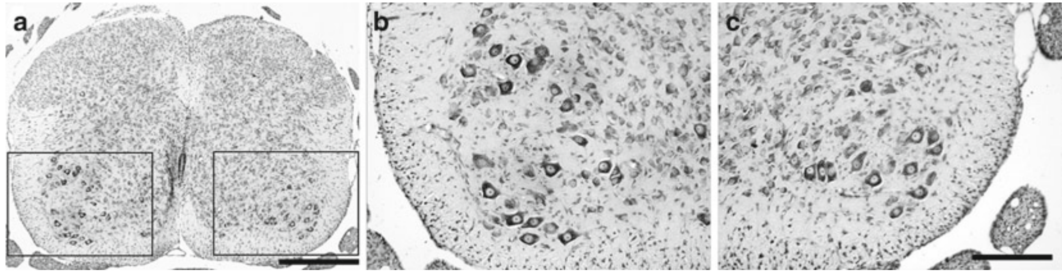

Fig. 3. Transverse sections of the lumbar enlargement (L4) of rats 5 days after sciatic transection performed at an age of 2 days postnatally. (**a**) Reduction of the number of motor neurons is observed in the right ventrolateral region (shown in (**c**)) as compared to the contralateral unlesioned side (shown in (**b**)). Paraffin-embedded sections stained with cresyl violet. Bar: 500 μm (**a**), 100 μm (**b**, **c**).

of counting the nucleoli of motoneurons ipsilateral and contralateral to the axonal injury. In general, we count nucleoli in 20 serial paraffin sections (5 μm) per animal. Specifically, the first section of every four is considered for counting. Then, a ratio of the total number of nucleoli in the lesioned side to that in the unlesioned side is calculated for each animal and used for further statistical analyses (17, 18) (see Fig. 3). Alternatively, the spinal cord may be subjected to stereological techniques for a quantitative approach, such as the "optical fractionator" (21). For this goal, we usually freeze the lumbar enlargement and obtain 40-μm serial sections. Subsequently, a random systematic uniform sampling is performed for the selection of sections used for counting and for the placement of a grid of known size in each sample. Then, an optical dissector, a three-dimensional counting probe, is placed in each point of intersection of the grid that falls in the region of interest. The total number of cells is given by the formula $C = \Sigma Q^{-} \cdot t/h \cdot 1/asf \cdot 1/ssf$, where C is the total number cells, ΣQ^{-} is the sum of cells counted in the optical dissector, t is the section thickness, h is the dissector height, asf is the ratio between counting frame and grid areas, and ssf is the ratio between sections used for counting and total number of sections. For details on the fractionator and other stereological tools, the reader is referred to an introductory review by Gundersen et al. (22).

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