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Cell Body-Motor Neuron Counts

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1. OBJECTIVE

This document describes methods and provides reference values for the quantitative and reliable measurement of pathology-relevant histological parameter of cell body-motor neuron counts in the SMA mouse.

2. SCOPE AND APPLICABILITY

The clinical features of Spinal muscular atrophies (SMAs) are basically caused by the progressive loss of alpha motor neurons in the anterior horns of the spinal cord, which leads to symmetrical weakness and atrophy of the proximal voluntary muscles of legs, arms, and eventually of the entire trunk during disease progression [1,2,3,4]. SMA is a neurodegenerative disease caused by mutation of the Survival Motor Neuron 1(SMN) gene. The disease is thought to be caused by abnormalities of motor neurons (MNs). Advances in transgenic technology have enabled investigators to study this disease in mouse models [5,6,7].

Motor neurons can be basically identified by evaluating location, size and morphology of ventral horn spinal neurons. However, the use of motor neuron-specific markers is crucial for proper identification, since not all large neurons within the ventral spinal cord are motor neurons. In addition, in diseased mice, motor neuron phenotype of may vary at different stages of degeneration. Therefore, it might not be easy to have a single protocol that covers various models and their time courses. Two separate protocols are listed on this Standard Operating Procedures (SOP). The first one is the use of Haematoxylin and Eosin (H&E) or thionine stainings, which are based on basic morph metric parameters of motor neurons. The other is the use of choline acetyl- transferase (ChAT) staining, which specifically stains motor neurons in spinal cord sections.

Since both protocols offer advantages and disadvantages, they should be better used simultaneously to avoid pitfalls and limitations in cell body-motor neuron counts.

3. CAUTIONS

The procedure requires careful surgical dissection of spinal cord from perfused animals. Sections need to be carefully cut without damaging tissues. In particular the segment levels of the spinal cord have to be carefully documented during preparation and tissue processing.

4. MATERIALS

- LSM510 (Carl Zeiss) confocal microscope, Lexica SP2 or SP5, or equivalent confocal microscope
- Light Microscope (Carl Zeiss, Leica or equivalent)
- Cryosection Machine: OTF Bright, Leica CM, or equivalent cryostats
- Microscope Slides: Thermo Scientific 76×26mm (Cat. No. 2600279)
- Tissue-Tek O.C.T: Sakura (Cat. No. 4583)
- Para-formaldehyde: Fluka (Cat. No. 76240)
- Triton X 100: Sigma (Cat. No. T8787)
- Alexa Fluor 488 or cy3 conjugate wheat germ agglutinin (WGA): Molecular Probes (Cat. No. W-11261; use 1mg/mL in PBS as 1000 x stock solution)
- FluorSaveReagent ™: Calbiochem-Novabiochem, San Diego, CA, USA (or equivalent).

Stock Solutions-PBS

- PBS (e.g. 7.2g Na2HPO4, 1.2g KH2PO4, 40g NaCl and 1g KCl; dissolved in 1L H2O)
- 4% PFA in PBS (prepared in batches by overnight stirring and stored in 50ml aliquots at 20°C).

Cryosectioning Stock – 1% aqueous Eosin-Y

Stock – 1% aqueous Phloxin B

Working Solutions – Eosin:

- 100ml stock Eosin
- 10 ml stock Phloxin B
- 780 ml 95% Ethanol
- 4 ml glacial Acetic Acid

Working Solution-Hematoxylin

Harris Hematoxylin, Sigma, HHS-32, 1 Liter

Working Solution-Lithium Carbonate 1.36%

- Lithium Carbonate, 47g
- dH_2O, 3500 ml

Working Solution-0.25% Acid Alcohol

- 95% Ethanol, 2578 ml
- dH_2O, 950ml
- HCl, 9ml

5. METHODS

5.1 Termination under protocol

Animals are sacrificed at different time-points depending on the clinical features of the considered model. In general, multiple time-points (at birth, clinical onset, pre-agonic stages) should be taken into consideration. Mice from each group are terminally euthanized by an overdose of pentobarbital (I.P.) and then transcardially perfused with 10ml cold 0.9% saline containing 2000 units/mL heparin after which the solution is replaced with 10mL cold 4% paraformaldehyde (PFA) in 0.1M PBS. Dissection is performed carefully preserving the anatomical integrity of the spinal cord tissue. This step is particularly relevant since the size of spinal cords from diseased SMA mice can be different and greatly reduced if compared to wt littermates. The same levels of spinal cord are then collected from each group and post-fixed in 4% PFA for 16-24 hours.

5.2 Cryosectioning

The tissues are cryoprotected in cold 30% sucrose (g/vol) made up in PBS, before processing. To achieve a thermal equilibration before cryosectioning, store the samples overnight in the -20°C freezer and place them into the cryostat for at least 20 minutes before further processing. Mount the sample on the round metallic mount of the cryostat with Tissue-Tek O.C.T. The knife should be pre-cooled to -20°C and the spinal cord sample to -24°C. Make 20 µm-thick sections and collect them on warm (RT) slides. Unstained slides are stored at -20°C.

5.3 Hematoxylin and Eosin Staining [8,9]

5.3.1 Deparaffinize in Xylene I and II and III (5 minutes)

5.3.2 Rehydrate

a. EtOH 100% (3 minutes)
b. EtOH 100% (3 minutes)
c. EtOH 95% (3 minutes)
d. EtOH 95% (3 minutes)
e. EtOH 70% (3 minutes)

5.3.3 Rinse in distilled water (5 minutes)
5.3.4 Stain in hematoxylin (6 minutes)
5.3.5 Rinse in running tap water (20 minutes)
5.3.6 Decolorize in acid alcohol (1 second)
5.3.7 Rinse well in tap water (5 minutes)
5.3.8 Immerse in Lithium Carbonate (3 Seconds)
5.3.9 Rinse in tap water (5 minutes)
5.3.10 Counterstain in Eosin (15 seconds)
5.3.11 Dehydrate
   a. EtOH 95 % (3 minutes) ***Discard after each use***
   b. EtOH 95% (3 minutes)
   c. EtOH 100 % (3 minutes)
   d. EtOH 100 % (3 minutes)

5.3.12 Clear in Xylene I and II (5 minutes)
5.3.13 Mount with Cytoseal in fume hood.
5.3.14 Images are taken by light microscope

5.4 Immunocytochemistry-ChAT staining

5.4.1 Bring the slides to room temperature
5.4.2 Place the slides in 0.3% Triton-X100 in PBS for 20 minutes (permeabilization)
5.4.3 Wash with PBS 3 times for 5 minutes each (e.g. in a staining beaker)
5.4.4 Blocking: 10%Normal Goat Serum (DAKO), 0.3% Triton X-100 in PBS
5.4.5 Incubate primary antibody in blocking solution at 4°C overnight. A primary antibody against choline acetyl transferase (ChAT, 1:200; Chemicon International, Temecula, CA) which is a well known marker for cholinergic motor neurons is used.
5.4.6 Wash 3 times for 10 minutes each with PBS
5.4.7 Secondary antibody in PBS 3hours at room temperature in dark: The ChAT antibody is visualized by the subsequent use of a secondary antibody conjugated to a fluorochrome (FITC or Cy5). As an alternative, use a biotinylated secondary antibody (3 hours at room temperature) followed by streptavidin conjugated to a Fluorochrome (rhodamin, Cy2, etc) for 2 hours at room temperature. This additional step may help identifying motor neurons with weaker ChAT immunoreactivity.
5.4.8 Wash 3 times for 10 minutes each with PBS
5.4.9 Allow the slides to dry for 5 minutes at room temperature
5.4.10 Add 2 drops of FluorSaveReagent ™ to the slides and mount the cover-slip. Avoid the formation of air bubbles. Place at 4°C for 30 minutes before viewing. LSM510 (Carl Zeiss) confocal microscope is used for taking images.

5.5 Thionine staining

5.5.1 Rehydrate sections from 5.2 (which are mounted on glass slides):
- a. EtOH 80% (3 minutes)
- b. EtOH 70% (3 minutes)
- c. EtOH 50% (3 minutes)
- d. distilled water (5 minutes)

5.5.2 Stain in thionine (0.1% in distilled water, 20 to 60 seconds)
5.5.3 Rinse in distilled water 2-3 times
5.7.4 Decolorize in EtOH 50%
5.5.5 Dehydrate:
- a. EtOH 70% 3 seconds
- b. EtOH 80% 3 seconds
- c. EtOH 90% 3 seconds
- d. EtOH 100% 3 seconds

5.5.6 Clear tissue in xylene (twice, in different vials, 5 minutes each)
5.5.7 Coverslip with DPX mountant for microscopy (BDH) in fume hood
5.5.8 Air-dry before light microscope examination

5.6 Motor neuron counts from paraffin embedded spinal cord sections [13, 14, 15]

The spinal cords are prepared from mice after perfusion with 4% freshly prepared paraformaldehyde or after being dissected and immersion-fixed in either Bouins or Carnoys solution, processed, paraffin-embedded, sectioned serially (7–10 µm), and stained with either thionin, cresyl violet, or hematoxylin and eosin. Motor neurons are counted in every 5th or 10th section through each population examined, and the totals are multiplied by 5 or 10, respectively to give an estimate of total cell numbers. Cell counts should be done blind as to the treatment condition of the embryo–postnatal animals (control vs experimental) using established methods that effectively eliminate the possibility of counting the same cell (healthy or pyknotic) twice[16].

6. EVALUATION AND INTERPRETATION OF RESULTS

When examining H&E or thionine stained sections always keep in mind that large spinal neurons projecting to supra-spinal centers (e.g., spinocerebellar neurons) may be located in the ventral horn and misinterpreted as motor neurons..When examinig ChAT immunoreacted sections keep in mind that the disease process in SMA may lead to the

down regulation of ChAT. Therefore, weaker ChAT stained motor neurons should be counted to avoid underestimating the number of motor neurons in SMA mice. A second motor neuron marker (CGRP, etc) could be used to confirm the motor neurons for counting. Serial sections must be cut at the same level of spinal cord for all groups. The size of the spinal cord in SMA mice may be greatly reduced if compared to that of control mice. Therefore, the number of cut sections must be always considered when counting the total number of motor neurons for a given spinal cord segment. In other words, the number of motor neurons per section is not a sufficient parameter to evaluate the total loss of motor neurons. Double-blind counting is necessary for all experiments.

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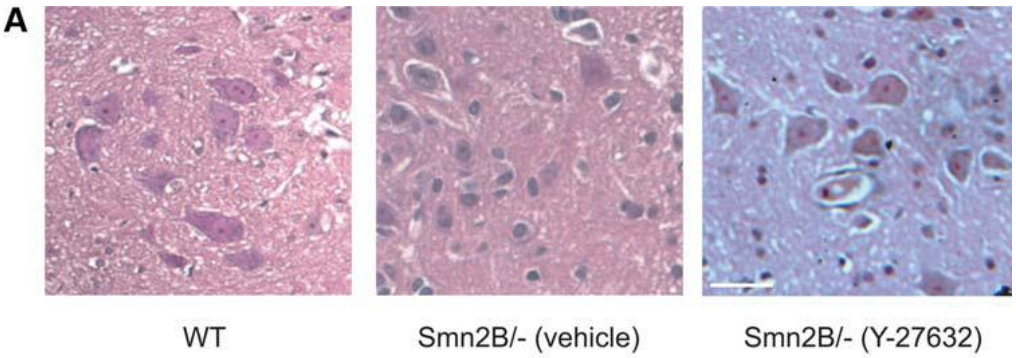
8. APPENDIX

8.1. Examples of H&E staining

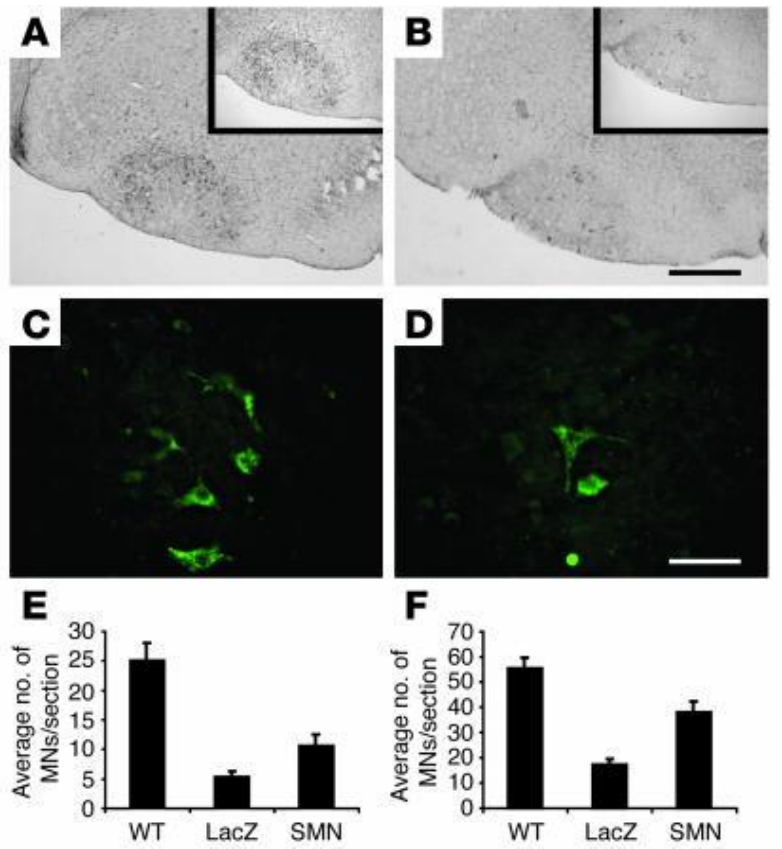


Y-27632 administration does not protect from motor neuron loss in Smn2B/2 mice

A. H&E staining of the motor neurons in the ventral horn region: untreated wild-type, vehicle-treated Smn2B/2, Y-27632-treated Smn2B/2 mice.
B. Quantification of the number of motor

(M. Bowerman et al. Human Molecular Genetics, 2010)

8.2 Examples of ChAT and CGRP staining



SMN gene replacement protects spinal and brain stem MNs in SMA mice.

ChAT: A. LV-SMN–treated mice, **B**. LV–Lac-Z–treated mice

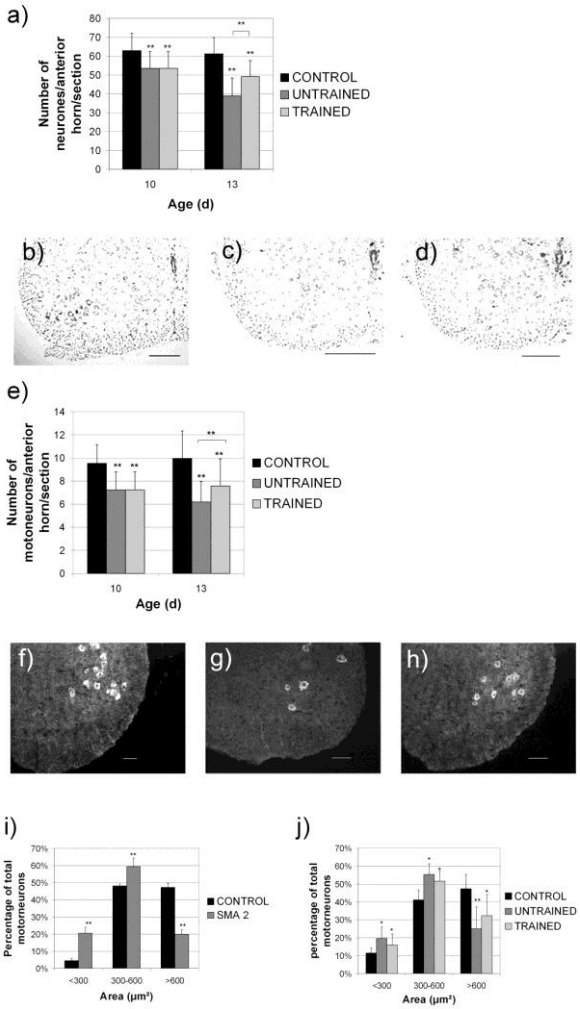
CGRP-positive MNs in lumbar spinal cord: C. LV-SMN–injected mice, **D**. LV-LacZ–injected mice

E. Cell counts of surviving lumbar spinal cord MNs (at end stage of disease):
WT (control), LV–Lac-Z-treated SMA mice, LV-SMN–treated SMA mice

F: Quantification of facial nucleus MNs (at end stage of disease):
WT (control), LV-LacZ–injected SMA mice, LV-SMN–injected SMA mice

(Azzouz et al. J Clin Invest. 2004)

8.3 Examples of Nissl and ChAT staining



Exercise-induced protection of neurons.
a: Quantification of surviving neurons in the ventral horn of the lumbar spinal cord (L1-L5) at 10 and 13 d of age.
b-d: **Nissl staining**.
f-h: **ChAT immunostaining** The number of motoneurons was determined in spinal cord removed from control (***f***), untrained (***g***), and trained (***h***) type 2 SMA-like mice. Scale bar, 50 µm.

(C. Grondard et al. J Neurosci., 2005)

8.4 An example where Paraffin embedded serial spinal cord sections have been used to count motoneurons in Smn+/- mice

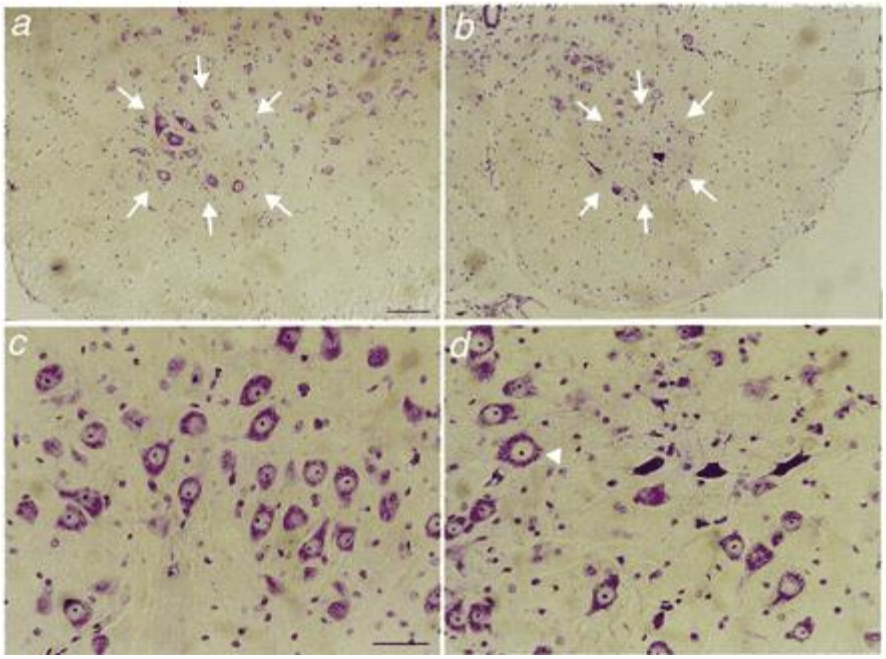


Figure 3. Morphology of facial and spinal motor neurons in *Smn*$^{+/+}$ and *Smn*$^{+/-}$ mice. (**a** and **b**) Spinal motor neurons in 6-month-old *Smn*$^{+/+}$ (a) and *Smn*$^{+/-}$ (b) mice: motor neurons in *Smn*$^{+/-}$ mice appear smaller and chromatolytic. Interneurons in the ventromedial part of the spinal cord are unaffected. The position of the ventrolateral motor neuron pool is indicated by arrows. (**c** and **d**) Facial motor neurons in 12-month-old *Smn*$^{+/+}$ (c) and *Smn*$^{+/-}$ (d) mice: a significant number of facial motor neurons appear smaller and chromatolytic, a few cells [arrowhead in (d)] are larger than motor neurons in the control animal, suggestive of hypertrophy. Bar in (a), 50 µm; bar in (c), 100 µm.

Table 1. Number of spinal motor neurons (L1–L6) in *Smn*$^{+/+}$ and *Smn*$^{+/-}$ mice

Age	*Smn*$^{+/+}$	*Smn*$^{+/-}$	Reduction
Postnatal day 1	3180 ± 166 (*n* = 4)	3119 ± 339 (*n* = 4)	Not significant
6 months	3164 ± 431 (*n* = 4)	1913 ± 239 (*n* = 4)	–40% (*P* < 0.05)
12 months	3229 ± 164 (*n* = 3)	1458 ± 70 (*n* = 3)	–54% (*P* < 0.05)

(S. Jablonka et al. Human Molecular Genetics 2000)