

Video Article

Clinical Testing and Spinal Cord Removal in a Mouse Model for Amyotrophic Lateral Sclerosis (ALS)

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder resulting in progressive degeneration of motoneurons. Peak of onset is around 60 years for the sporadic disease and around 50 years for the familial disease. Due to its progressive course, 50% of the patients die within 30 months of symptom onset. In order to evaluate novel treatment options for this disease, genetic mouse models of ALS have been generated based on human familial mutations in the SOD gene, such as the SOD1 (G93A) mutation. Most important aspects that have to be evaluated in the model are overall survival, clinical course and motor function. Here, we demonstrate the clinical evaluation, show the conduction of two behavioural motor tests and provide quantitative scoring systems for all parameters. Because an in depth analysis of the ALS mouse model usually requires an immunohistochemical examination of the spinal cord, we demonstrate its preparation in detail applying the dorsal laminectomy method. Exemplary histological findings are demonstrated. The comprehensive application of the depicted examination methods in studies on the mouse model of ALS will enable the researcher to reliably test future therapeutic options which can provide a basis for later human clinical trials.

Video Link

The video component of this article can be found at <https://www.jove.com/video/3936/>

Protocol

Animals were purchased from Jackson Laboratory (# 002726) ¹. They are clinically scored and subjected to a test of motor function (rotarod test) and of muscular strength (hanging wire test). All these tests and the later killing of the animals in order to prepare the spinal cord have been performed in very close accordance to the local guidelines for proper conduct of animal experiments.

1. Clinical Score

Apart from assessment for body weight mice are examined for signs of motor deficit with the following 4 point scoring system ²:

4 points: normal (no sign of motor dysfunction)

3 points: hind limb tremors are evident when suspended by the tail

2 points: gait abnormalities are present

1 point: dragging of at least one hind limb

0 point: symmetrical paralysis, inability to right itself or loss of 20% of maximum body weight; in this case the animals are immediately euthanized and the experiment is terminated

2. Tests of Motor Function and Muscular Strength

Hanging wire

This test is used to assess muscular strength ^{3,4}. All animals perform this test at least one or two days after the rotarod test. Each mouse is placed on a custom-made wire lid with intervals of 0.8 cm and cautiously turned upside down, 60 cm above a straw covered bottom. After training for three consecutive times of at least 180 s the latency to fall is measured. Each mouse is given up to three attempts to hold on to the inverted lid for a maximum of 180 s and the longest period is recorded.

Rotarod Test

The rotarod apparatus (Ugo Basile, Comerio, Italy) was used to measure motor coordination, balance and motor learning ability^{3,4}. A good performance requires a high degree of sensorimotor coordination. The machine should be placed in a calm and non-disturbing environment to avoid distractive stimuli for the tested animal. It consists of a computer-controlled motor-driven rotating spindle and five lanes for five mice. Falls of the mice are detected automatically by pressure on a plastic plate at the bottom. After training for three consecutive times of at least 180 s at a constant speed of 15 r.p.m. the time for which an animal can remain on the rotating rod is measured. Each animal undergoes three trials and the longest latency without falling is recorded. The time of 180 s is chosen as cut-off time because the majority of significant differences in motor coordination are detected in this time frame.

3. Spinal Cord Preparation

- a. Animals are killed by CO₂ insufflation in accordance with the local guidelines and are immediately perfused transcardially with PBS solution followed by a 4% paraformaldehyde solution.
- b. In order to prepare the spinal cord of the sacrificed mouse, the animal is placed onto an operation table and the four limbs are fixed on the side in order to expose the back side of the mouse.
- c. A short wash with a 70% ethanol solution cleans the site of dissection and flattens the hair coat.
- d. Then the skin is incised with a sharp scalpel in the median line. In order to facilitate cutting the skin is stretched to both sides. If leg muscles shall be prepared, their skin has also to be incised.
- e. After the skin incision is completed, it is pulled aside with a pair of tweezers to expose the underlying superficial fascia of the body.
- f. The musculature of the neck and the nuchal ligament have to be removed and are carefully prepared. Be careful not to incise too deeply and lesion the spinal cord. The shoulder muscles can also be removed in order to better expose the spinal column.
- g. Then the paravertebral muscles are removed from the entire spinal column.
- h. In order to open the spinal column several laminectomies have to be performed. One should start from the upper cranial part at the site of the atlanto-occipital joint.
- i. It is easiest to remove the fixation of the upper two limbs and overstretch the neck to be better able to perform the laminectomy of the first vertebrae. These are pulled away without touching the exposed cervical spinal cord.
- j. More vertebrae are removed by first transecting the vertebral arches at both sides with angled scissors and then pulling on the dorsal processes. Remaining lateral parts of the vertebrae should be removed to facilitate later complete removal of the spinal cord.
- k. An anatomical landmark of the lumbar spinal cord is the intumescence that is also present in the cervical spinal cord.
- l. Having finished the laminectomy of the entire spinal cord, make sure that you also transect all ventral roots and release the spinal cord from the dura mater of the meninges.
- m. Then the cervical spinal cord is cut cranially and you start to remove the spinal cord.
- n. Finally the spinal cord is also cut at the distal cauda-equina to be completely released.
- o. Ultimately, the spinal cord is placed into a postfixing solution (e.g. 4% paraformaldehyde) overnight and can be further processed. We usually cryosection the spinal cord to prepare it for immunohistological analysis.

4. Representative Results

The technique of spinal cord preparation represents the focus of this video article. It is an essential prerequisite for later tissue sectioning and ultimately for immunohistological analysis of spinal cord sections. As an example of a final result, an immunohistochemical workup of the anterior horn region of the mouse lumbar spinal cord of a wildtype (wt) and of a SOD G93A transgene (tg) mouse is demonstrated. Motor neurons can be identified with a primary anti-ChAT antibody and subsequent fluorescent labeling with a secondary Cy3 antibody. In addition, a nuclear counter-stain with DAPI (4,6-diamidino-2-phenylindole) has been performed (**Figure 1**).

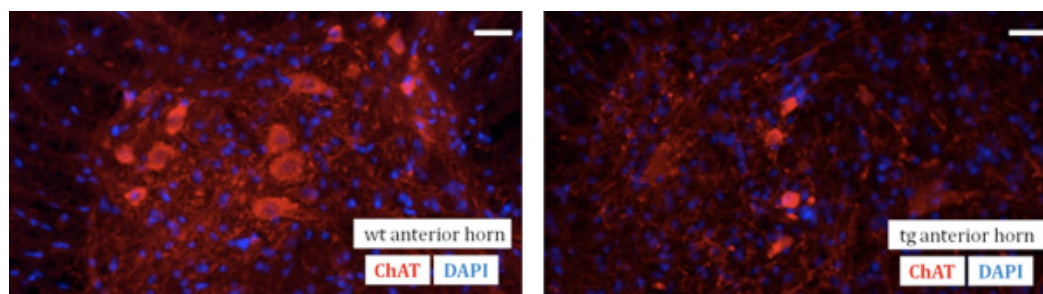


Figure 1. Fluorescent photomicrographs visualizing the immunodetection of motoneurons with anti-ChAT antibody (red) and cellular nuclei counter-stain by DAPI (blue) in the mouse lumbar spinal cord anterior horn of a wildtype (wt) (on the left) and of a SOD G93A transgenic (tg) (on the right) mouse at the age of 130 days. Scale bar: 40 μ m.

As the immunohistochemical analysis of the SOD G93A mice is not the primary scope of this article please consult the original publication in which these transgenic mice have been characterized and more recent ones which study therapeutic approaches for further reference^{1,5,6}. If therapeutic effects shall be differentiated on the immunohistological level clearly defined quantitative evaluation algorithms should be applied supported by a stereological software (for example see⁷).

Discussion

The SOD1 (G93A) genetic mouse model is a valuable animal model to study the disease course of progressive motoneuron loss comparable to human amyotrophic lateral sclerosis⁸. A variety of different treatment paradigms have been evaluated in this model and represent a basis for later testing in human clinical studies⁸⁻¹⁰. In order to be able to detect significant differences in an experimental treatment study in these mice, it is of eminent importance to include at least 24 litter-matched gender-balanced mice of the same genetic background and follow a double-blinded design⁸. Comparable to human clinical studies, a single uniform endpoint criterion should be chosen. Here, the most commonly used one is the inability of the animal to right itself in 30 seconds after being placed on its side. If this criterion is reached the animal is sacrificed and its life time is recorded as time of survival.

Treatment studies can be started at a presymptomatic clinical state (e.g. at day of life (DOL) 50 or even on DOL 30) when the animals do not yet exhibit any sign of motor dysfunction and would be scored with 4 points in our clinical scoring system. Another possibility consists in a symptomatic treatment approach which should start when the first clinical symptom appears and hind limb tremors are evident when the animal is suspended by the tail. This is defined as disease onset and this initial disease stage is graded with the clinical score 3. Significant impairments in tests of motor function and of muscular strength mostly occur only more than two to four weeks later. As on average the animals reach the first clinical disease stage around DOL 80, some researchers start their symptomatic treatment for all animals at this time point in a simplified approach. In both settings it is obligatory to monitor disease progression. This should be started at the time of treatment initiation for presymptomatically treated animals (DOL 50 or DOL 30, respectively) or at DOL 70 for symptomatically treated animals. Monitoring of disease progression includes a twice weekly determination of body weight, clinical neurologic scoring and tests of motor function and muscular strength. If the animals reach clinical score 1 (dragging of at least one hind limb) we recommend to perform daily determination of body weight and clinical monitoring in order not to miss the clinical score 0 point (symmetrical paralysis, inability to right itself or loss of 20% of maximum body weight) when the animals have to be immediately euthanized and the experiment is terminated. It is recommended that a first-time examiner is guided by an experienced animal researcher who helps to detect also subtle clinical signs of progression. The clinical 4 point-scoring system after Weydt *et al.*² is well established in the field and is a reliable system using criteria which can be clearly differentiated. A more subtle differentiation of clinical symptoms would be less clear and highly examiner dependent.

In order to detect behavioural deficits a variety of test paradigms is available. Most authors favor the rotarod test that evaluates the ability of the animal to run on a rotating cylinder^{4, 11}. In a study evaluating the significance of behavioural tests in the SOD1 G93A mouse model the rotarod test proved to be very sensitive in detecting significant differences between wildtype and transgenic mice as early as from week 16 of age on¹¹. Possible modifications include running at a constant speed or an accelerated speed run. In any case, the animals have to be trained before the first valid testing because some may need more training than others to obtain a basic level of motor coordination. A limitation is represented by differently motivated animals to perform this task. This, however, can be compensated by repeated tests of at least three times per examination day. Another motor behavioural test which is very sensitive in detecting early motor deficits is the footprint analysis¹¹. However, it is quite laborious because the animals have to be motivated to run over a gangway after having their feet dipped into a reservoir of paint. The quality of the footprint can vary to a large extent and a software-aided analysis is difficult. Therefore, we prefer the rotarod test for the evaluation of motor coordination.

The hanging wire test evaluated muscular strength of the limbs. It is a rather crude test that best detects early muscular deficits a few weeks after initiation of disease⁴. However, it is very easy to perform and the entire testing apparatus can be easily constructed. A more elaborate test for muscle strength is the use of a force transducer¹². Here, the mouse is prompted to grab a bar connected to a force transducer with either its hind or its fore paws. Other functional tests that have to be considered include the measurement of the running wheel distance or even the evaluation of open-field activity^{3, 13}. However, in our experience the combination of the rotarod and the hanging wire test proved to be most sensitive, easily practicable and time efficient for the evaluation of the SOD1 G93A mice. Overall, a preclinical study in animals should be designed very carefully and should follow basic principles of a human clinical trials as described in the CONSORT guidelines (www.consort-statement.org)¹⁴. Only then, the use of animals for preclinical research can be justified and the results may ultimately lead to a successful translation into a human clinical application.

These clinical and behavioural findings should always be correlated to an analysis of the pathology of the neuromuscular unit including spinal cord motoneuron, axon and neuromuscular junction¹⁴. Here, a high-quality analysis of CNS spinal cord pathology is a prerequisite for the interpretation of effects in survival or disease progression. Because thorough tissue fixation is critical, the perfusion of the animals with a 4% paraformaldehyde containing PBS-solution should be standardized well. In order to be able to carefully remove the spinal cord a dissection table should be installed together with a fixed operation microscope and fine surgical instruments have to be available (see list of surgical instruments below). After the entire spinal cord has been removed, it can be processed to the appropriate sectioning technique (e.g. vibratome or cryotome) and can finally be subjected to immunohistological analysis. Here, basic evaluation parameters are the numbers of spinal cord motoneurons and activated or infiltrating glial cells^{10, 15}. Depending on the original research question, additional immunohistochemical markers like SOD aggregates or CNS endothelium integrity can be evaluated. Furthermore, peripheral nervous system disease pathology can be evaluated by examination of peripheral nerve axons and of neuromuscular junctions. Only the combination of both clinical and immunohistochemical analyses of CNS and PNS provide a thorough view of overall ALS pathology that has to be correlated with the clinical findings.

Disclosures

No conflicts of interest declared.

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