Innovative Methodology

Long-term measurement of muscle denervation and locomotor behavior in individual wild-type and ALS model mice

Turgay Akay
Department of Neurological Surgery, Center for Motor Neuron Biology and Disease, Columbia University Medical Center, New York, New York

Submitted 12 July 2013; accepted in final form 28 October 2013

Akay T. Long-term measurement of muscle denervation and locomotor behavior in individual wild-type and ALS model mice. J Neurophysiol 111: 694–703, 2014. First published October 30, 2013; doi:10.1152/jn.00507.2013.—The increasing number of mouse models of human degenerative and injury-related diseases that affect motor behavior raises the importance of in vivo methodologies allowing measurement of physiological and behavioral changes over an extended period of time in individual animals. A method that provides long-term measurements of muscle denervation and its behavioral consequences in individual mice for several months is presented in this article. The method is applied to mSod1G93A mice, which model human amyotrophic lateral sclerosis (ALS). The denervation process of gastrocnemius and soleus muscles in mSod1G93A mice is demonstrated for up to 3 mo. The data suggest that as muscle denervation progresses, massive behavioral compensation occurs within the spinal cord that allows animals to walk almost normally until late ages. Only around the age of 84 days is the first sign of abnormal movement during walking behavior detected as an abnormal swing movement during walking. Additionally, this method can be used with other mouse models of human diseases, such as spinal cord injury, intracerebral hemorrhage, Parkinson’s diseases, and spinal muscular atrophy.

muscle denervation; ALS; walking; H-reflex

THE MOUSE HAS BECOME THE STANDARD model for research aiming to understand the mechanisms of human diseases. This fact is fueled by the availability of diverse mouse models for injuries, such as spinal cord injury and intracerebral hemorrhage (Rynkowski et al. 2008; Steward et al. 1999), and mutant mouse lines modeling human degenerative diseases, such as Alzheimer’s disease, Parkinson’s disease, spinal muscular atrophy (SMA), and amyotrophic lateral sclerosis (ALS; Ebadi et al. 2005; Wong et al. 2002). The availability of these invaluable models of human diseases raises the importance of in vivo methodologies allowing the measurement of physiological and behavioral changes that occur over extended time periods in individual animals. This article demonstrates a method that can be used to measure muscle denervation and its behavioral consequences in individual mice for up to 3 mo, which is essential for research aiming to understand disease progression in degenerative diseases, such as ALS.

ALS is a human disease with adult onset where motor neurons (MNs) die progressively, leading to paralysis and, in most cases, death (Kanning et al. 2010). Considerable knowledge regarding ALS disease progression has been gained by using an array of methods to study ALS mouse models. These range from histological approaches, in vitro physiological techniques, through to behavioral studies. Even though the MN death is the core of the paralysis, there is strong evidence that non-cell-autonomous mechanisms play a key role (e.g., toxic astrocytes; Di Giorgio et al. 2007; Nagai et al. 2007). Previous work using ALS model mice (mSod1G93A) also suggests that the degree of paralysis exceeds the extent of MN loss (Chiu et al. 1995), and electrophysiological changes arise in MNs before any pathological changes occur in the spinal cords (Quinnan 2011). The non-cell-autonomous mechanisms point to the importance of physiological measurements, where the MNs reside in their natural environment.

Documentation of physiological changes occurring in MNs in their native environments requires in vivo measurements of MN physiology in individual ALS model animals starting from any presymptomatic early ages throughout the disease progression until the end stage of the disease. Electromyographic (EMG) activity measurements from multiple muscles in vivo during free behavior in mutant mice have been performed in several projects (Akay et al. 2006; Leblond et al. 2003; Pearson et al. 2005; Tysseling et al. 2013). In one of these studies, EMG recordings were performed regularly for up to 5 wk on the ALS mouse model mSod1G93A (Tysseling et al. 2013). However, Tysseling et al. (2013) did not provide any measure of muscle denervation in parallel with behavioral observations, and their measurements were limited to just 1 mo. To have full benefit of behavioral measurements, one has to provide physiological parameters that correlate with the muscle denervation in these animals. In the past, physiological measurement of muscle denervation in vivo has been accomplished by measurement of compound muscle action potentials (CMAP; Maathuis et al. 2013).

In this article, a method is presented that allows continuing measurements of CMAP (Mmax) from gastrocnemius (Gs) and soleus (Sol) muscles for several months in parallel with kinematic parameters and EMG activity pattern during walking. To demonstrate the effectiveness of this technique, I applied it to the ALS mouse model, mSod1G93A, that has been previously described in detail by histological methods (reviewed in Kanning et al. 2010). The data suggest that loss of MNs, as the disease progresses, is compensated in such a way that the EMG pattern is normal until the degeneration of muscle innervation exceeds a certain degree.

METHODS

All surgical procedures and behavioral measurements were performed in accordance with the National Institutes of Health Guide for
Animal model. To characterize the muscle denervation and changes in locomotor behavior in individual mice that mimic ALS, a transgenic mouse line expressing the mutant form of Cu/Zn superoxide dismutase 1 enzyme (substitution of Gly93 with Ala; mSod1<sup>G93A</sup>) was used. In total, five wild-type mice and five mSod1<sup>G93A</sup> mice (low expressor; Gurney et al. 1994) were used for this study. It has been shown previously that the mSod1<sup>G93A</sup> mice develop stereotyped phenotypes resembling the symptoms in ALS patients. The phenotypes observed include adult onset of muscle denervation followed by MN death and subsequent paralysis and finally death at ages ranging from postnatal day 140 (P140) to 180 (P180; Gurney et al. 1994).

Construction of the electrodes. The fabrication of the EMG recording electrodes was previously described in great detail (Pearson et al. 2005). The fabrication of the nerve cuff electrodes was as following.

The cuff was fabricated by using 1–2 mm length of AlliedSil Silicone Tubing (implant grade, P03; Allied Biomedical, Ventura, CA). Two 9-cm long wires (stainless steel, 7-strand, PFA, 0.001 in. bare, 0.005 in. coated; A-M Systems, Sequim, WA) were cut. From these 9-cm wires, insulation was removed 8 mm from 1 side and 1 mm from the other side. The 8-mm bare regions of both wires were driven in parallel through the silicone tube by using a 27-gauge needle as illustrated in Fig. 1, A and B. Between both wires, a suture (6-0 Sofsilk) was driven so that ~2 cm of suture would extend on both entry and exit sides of the tube. The tube was covered evenly with silicone adhesive so all of the exposed bare wires were covered and the insulated part of the wires stayed parallel to each other and the tube (Fig. 1B). The tube was left overnight for the silicone adhesive to cure. The next day, when the silicone adhesive was cured, the part of the tube between the entry and exit of wires and suture was cut lengthwise (Fig. 1, B and C). The cuff electrode and 3 EMG recording electrodes were finally attached to the headpiece pin connector (fe-
male, SAM1153-12; DigiKey Electronics, Thief River Falls, MN) as previously described (Akay et al. 2006; Pearson et al. 2005; Fig. 1D).

Implantation of the electrodes. All surgeries were performed in aseptic conditions and on a warm water-circulated heating pad maintained at 42°C. The animal was anesthetized with isoflurane (5% for induction, 2% for maintenance of anesthesia), and the hind legs and the neck were shaved. Small incisions were made on the shaved areas (neck and on the level of the muscles to be implanted), and each bipolar EMG electrode and the nerve cuff electrodes were led under the skin from the neck incision to the leg incisions. The muscles that were recorded in these experiments were the vastus lateralis (VL), lateral Gastroc, Sol, and tibialis anterior (TA). The implantation of the EMG electrodes was previously described in detail (Akay et al. 2006; Pearson et al. 2005). For implantation of the nerve cuff electrodes, the distal end of the cuff electrodes was moved next to the tibial nerve. The tibial nerve was gently placed inside the tube and secured by knotting the 6-0 sutures at the cuff. The insulated wires close to the cuff were sutured on the surface of the surrounding fascia. The incisions on the legs were closed, and the headpiece was stitched to the skin near the neck incision. After each surgery, 0.1 mg/kg body wt buprenorphine (analgescic) was injected subcutaneously while the animals were still under anesthesia to avoid pain caused by the surgery. The mice were held under close observation for at least 1 wk. Any handling of the mice was avoided before the mice were fully recovered (~10 days). Right after the surgeries, all animals were able to care for themselves, that is, eat and drink.

Data collection. After animals fully recovered (in 10 days), the first recording sessions were performed. For this purpose, the mice were briefly anesthetized with isoflurane, and custom-made, three-dimensional reflective markers (~2-mm diameter, cone shaped) were glued onto the shaved skin at the level of the iliac crest, hip, knee, ankle, paw, and tip of the fourth digit of one hind leg as previously described (Akay et al. 2006; Pearson et al. 2005). The anesthetic was discontinued, and the mice were placed on a treadmill (Model 802; Zoological Institute, University of Cologne, Cologne, Germany) and connected to the amplifier (Model 102; Zoological Institute, University of Cologne, Cologne, Germany). After the animal fully recovered from the anesthesia, the treadmill was turned on at the speed of 0.2 m/s. The walking of the mice was recorded from the side by using a Photonfocus monochrome high-speed camera system (Systematic Vision, Ashland, MA) at 250 frames per second. The EMG data were simultaneously recorded (sampling rate: 10 kHz) by using Power1401 interface and Spike2 software (Cambridge Electronic Design, Cambridge, United Kingdom).

After recording of walking behavior, the tibial nerve was stimulated by delivering 0.2-ms current impulses at 1-Hz frequency at variable current strength (50–900 μA) while the EMG response at GS and Sol was recorded in resting animals. One-hertz stimulation frequency was chosen because at this rate no rate-dependent depression of the M-wave amplitude was observed in control or mutant animals. The electrical currents were delivered with a Biphasic Stimulus Isolator (DS4; Digitimer, Hertfordshire, United Kingdom) controlled by Spike2 and Power1401. After the nerve stimulation experiments were finished, the mice were placed in their cages for rest until the next recording session in 1 wk.

Data analysis. The kinematic data describing angular joint movements were obtained by automatically tracking the reflective markers from the video files by using Peak Motus Motion Analysis software (Vicon, Centennial, CO). The kinematic parameters were then imported to the Spike2 files that contained the EMG data in a way that the kinematic and EMG data were synchronized with a custom-written Spike2 script. The effective sampling rates in the final files were not altered, that is, 250 Hz for kinematic data and 10 kHz for the EMG data. Furthermore, the results from the nerve stimulation experiments were also analyzed by using Spike2 software. Statistical comparisons (ANOVA and Student’s t-test) were made by using Excel (2010, version 14).

RESULTS

In total, five wild-type mice were chronically implanted with three EMG recording electrodes into different combination of muscles (VL, TA, GS, and/or Sol) and one nerve stimulation electrode to the tibial nerve (Fig. 2A). Following a 10-day recovery period, brief current injections (0.2 ms) were applied to the tibial nerve, and the electrical response was recorded from the GS and/or the Sol (Fig. 2B). On the same day, the EMG activity pattern and kinematic parameters of the leg movement were obtained while animals were walking freely on the treadmill.

Examples of Hoffmann’s reflex (H-reflex) recordings from wild-type mice are illustrated in Fig. 2. When the tibial nerve was stimulated with the lowest current, the first axons to respond to the stimulation with the generation of an action potential were those of MNs and the primary muscle spindle afferents. The action potential generated by the MN axon traveled proximally to the spinal cord and distally toward the muscles. The action potential that traveled distally directly excites the muscle fibers, which responded with an action potential that was captured as the M-wave in GS and Sol EMG recordings (delay: <1 ms; Fig. 2B). The proximally travelling action potential normally arrives at the MN somata and with low probability returns back to the periphery, causing another motor response in the muscle with larger delay, which is called the F-wave (Magladery and McDougall et al. 1950). Furthermore, the action potential in the primary afferents (group Ia) traveled back to the spinal cord and excited the MNs through their largely monosynaptic connection to homonymous MNs. Therefore, MNs generated another action potential arriving at the muscles with a larger delay, and muscle fibers generated another response. This response is recorded as the H-wave and has a larger delay than the M-wave but similar to F-wave (2–3 ms; Fig. 2B). This spinally mediated response is known as H-reflex (Knikou 2008; Zehr 2002).

Gradual increases in stimulation strength recruited growing number of fibers in the muscles, recorded as increasing M- and H-wave amplitudes (Fig. 2, C and D). At low stimulation strength (≤110 μA for GS in Fig. 2C and ≤60 μA for Sol in Fig. 2D), no axons were activated, resulting in no M- or H-waves in the recorded muscles. After threshold was reached, M- and H-waves gradually grew as stimulation strength increased. After certain stimulation strength, M-wave plateaued (Mmax), indicating the activation of all motor units. H-wave, in contrary, the threshold for the M- and H-wave was in general the same in mice and sometimes even lower for the M-wave than for the H-wave. This is presumably due to the size difference of axons of MNs and primary afferents, which presumably is not large enough to be stimulated at different stimulation strength. Second, in humans, the H-wave normally decreases, whereas the M-wave increases and disappears when the Mmax is reached. In the present experiments, the H-wave decreased slightly or remained at constant amplitude when Mmax occurred but never disappeared. This is presumably
because at these higher stimulation intensities, the F-waves were mixed with the H-wave, thus masking the abolition of the H-waves. A similar observation was previously described during H-reflex measurement in rats (Gozariu et al. 1998). Additionally, this later H/F-wave response might also be contaminated by MN activation with very slow conduction velocities, making it difficult to perform reliable H-wave amplitude measurements, this later H/F-wave response might also be contaminated by MN activation with very slow conduction velocities, making it difficult to perform reliable H-wave amplitude measurements. Therefore, H-wave amplitude measurements will not be considered further.

In degenerative diseases, such as ALS, the $M_{\text{max}}$ recordings have to be performed in a continual way to measure the muscle denervation process that progresses slowly as the animal ages. To test the ability of this method to fulfill this requirement, long-term recordings of $M_{\text{max}}$ were performed in wild-type mice weekly for an extended period of time (Fig. 3). The average $M_{\text{max}}$ in all five wild-type animals varied between 12 and 28 mV for Gs and slightly increased over time as the mouse aged from P47 to P140 (ANOVA: $P < 0.001$ for all 5 animals; Fig. 3, A and B). For the Sol muscle, the $M_{\text{max}}$ varied between 2 and 4 mV, which had a slight tendency to increase over time as the mouse aged from P77 to P162 (ANOVA: $P < 0.001$). This feature of the $M_{\text{max}}$ recordings is very important for its application to degenerative disease models because it reflects the activity of the total number of muscle fibers activated by the MNs through its monosynaptic connection, generating an action potential in the MN that propagates to the muscle, and the muscle generates a 2nd response with larger delay (H-wave). C: dependence of M- and H-waves on stimulation strength is illustrated as 4 examples of average EMG recordings from Gs muscle ($n = 50$ stimuli, traces averaged around stimulation onsets indicated as vertical dashed lines) on the left and a graph showing dependence of average ($\pm$SD) M- and H-waves as a function of stimulation strength on the right. Notice that currents <100 $\mu$A do not generate any response in the Gs muscle. Once threshold is reached (110 $\mu$A), the M-wave can be recorded, and its peak-to-peak (PTP) amplitude grows with increasing stimulation strength. Finally, M-wave amplitude plateaus at a maximum ($M_{\text{max}}$). Amplitude of H-wave is always smaller, and after reaching the maximum amplitude, it decreases slightly. In this figure, the H-wave is labeled as H/F-wave because of the possible contamination of H-wave with F-wave responses (see text). D: as in C but EMG recordings from the Sol muscle. Data in B–D derived from different wild-type animals. TA, tibialis anterior; amp, amplifier.
movements are very similar except for the Gs activity. Notice that Gs activity gradually increased until the mouse reaches age P61 and remains constant in the following recordings. The VL recording was lost after P94. These recordings demonstrate that, together with weekly $M_{\text{max}}$ measurements, kinematic parameters and EMG muscle activity patterns can be recorded during free walking in the same animal at the same recording session.

To demonstrate the suitability of this method in the context of ALS, I applied it to four mice of the ALS mouse model, mSod1G93A. An example of average $M_{\text{max}}$ measurements at five different ages from these animals is illustrated in Fig. 5A. Notice that even though the $M_{\text{max}}$ at P70 was in the same range as wild-type mice (compare with Fig. 3A), it decreased at P124. At P131, the $M_{\text{max}}$ is increased up to the P97 level, but at P152 it dropped again. The changes in $M_{\text{max}}$ are illustrated in the graphs in Fig. 5, B and C, for the Gs and Sol, respectively. Overall, the $M_{\text{max}}$ recorded from Gs and Sol of the mutant animals showed a general tendency to decrease as the mice aged (ANOVA: $P < 0.001$ for all animals and both Gs and Sol recordings). Notice that $M_{\text{max}}$ in Gs changed drastically (ranges:

during walking at 0.2 m/s that include three swing phases (indicated by bursts of activity in TA recordings) at three different ages in the same wild-type mouse. Even though the electrodes were implanted when this particular mouse was P59, and the recordings have been performed every week for months, the M-wave amplitudes remained stable and the activity pattern very similar. Average (aligned to the onset of the stance phase) angular joint movements synchronized with rectified and band-pass filtered EMG recordings from another mouse are illustrated in Fig. 4B. In this particular mouse, the electrodes were implanted at age P33, and EMG recordings from Gs, VL, and TA synchronously with kinematic measurements were started at age P48 and continued until the mouse was P115. Overall, the EMG patterns and the angular joint

Fig. 3. Consistent recordings of $M_{\text{max}}$ for > 2 mo in wild-type mice. A: 3 averaged $M_{\text{max}}$ EMG responses (average of 50 sweeps for each trace, $n = 50$) to 400 $\mu$A at 3 different ages in the same wild-type animal. Slight increase in the $M_{\text{max}}$ is typically observed at these ages (ANOVA: $P < 0.001$ for each animal). B: graph illustrating average (±SD, $n = 50$) $M_{\text{max}}$ recorded in Gs muscles at 1-wk intervals from 4 different animals. C: as in B but recordings obtained from Sol muscle of 1 wild-type animal. In Sol muscle, the $M_{\text{max}}$ does not increase over time at these ages. The diamond on the very left in B and C represents the pooled average (±SD) of $M_{\text{max}}$ recorded from all wild-type animals at all days. WT1–5 indicate the number of specific wild-type animal. WT1–5 indicates data from the same wild type (WT1). The averages of 22 to 66 steps (22 $< n < 66$) are triggered by 10.220.32.246 on October 8, 2016 http://jn.physiology.org/ Downloaded from

Fig. 4. Consistent recordings of the locomotor pattern and angular joint movements for > 2 mo in wild-type mice. A: EMG activities of the TA, Sol, and Gs muscles during a walking sequence at 0.2 m/s that includes 3 swing phases (indicated by TA activity) at 3 different ages from an individual wild-type mouse (WT3 in Fig. 3B). Note the consistent pattern of activity during walking at different ages. B: average angular joint movement of the hip, knee, and ankle joints together with rectified and band-pass filtered EMG activities during walking at 0.2 m/s at different ages in the same wild-type animal (WT1). The averages of 22 to 66 steps (22 $< n < 66$) are triggered around the beginning of stance phase (vertical lines) at 9 ages for the same wild-type mouse. Vastus lateralis (VL) recording was lost after P94. The angular joint movements and EMG activity profiles vary to an extent, but once the animal reaches P61, the variability declines.

J Neurophysiol • doi:10.1152/jn.00507.2013 • www.jn.org
with wild-type values. In contrast, the M\text{max} values measured for Mut3 and P\text{in Gs in mSod1G93A mice (Mut1 and Mut2 in Fig. 5 but the recordings were obtained from Sol muscle of 2 mSod1G93A animals.}

The M\text{max} increases gradually until P90 but then stays constant as the disease progresses. The diamonds on the very left in graphs in Fig. 6, A and B. Furthermore, comparison of the threshold current and current for half-maximum and maximum M-wave shows no difference between wild-types and mSod1\textsuperscript{G93A} when the mSod1\textsuperscript{G93A} mice are younger than P100 but are significantly higher when they are older than P100 (Fig. 6C). This indicates that when the mSod1\textsuperscript{G93A} mice are younger than P100, the excitability of their MNs is not different from wild-type MNs but is less when they are older than P100.

In ALS, fast fatigable motor units involving MNs with lower activation thresholds in nerve stimulation experiments are more susceptible to denervation and MN death than slower

18.4–1.4 mV) as the disease progressed, whereas it varied around an average of 1.9 mV (±0.5 mV) in Sol (ranges: 2.4–1.6 mV).

Generally, all average M\text{max} values obtained from mSod1\textsuperscript{G93A} mice were significantly lower than the average M\text{max} values measured from all wild-type mice for Gs and all but 1-day measurement for Sol. In two out of three M\text{max} measurements in Gs in mSod1\textsuperscript{G93A} mice (Mut1 and Mut2 in Fig. 5B), the first average amplitude of the first (for Mut3) and the first two averages for Mut1, although significantly smaller (P < 0.001 for Mut3 and P < 0.01 for Mut1), were in comparable ranges with wild-type values. In contrast, the M\text{max} values measured from Gs in Mut2 were significantly lower than the wild-type values already at the 1st recording day when this mouse was P70.

The M-wave data suggest that MN excitability is not affected in mSod1\textsuperscript{G93A} mice at ages younger than P100 but is decreased after P100 (Fig. 6). The threshold current to elicit an M-wave and the current at the half-maximum and maximum M-waves are plotted as a function of age for Gs (Fig. 6A) and Sol (Fig. 6B) for wild-type and mSod1\textsuperscript{G93A} mice. Notice that all of these parameters do not change with age except for the threshold current to elicit M-wave in the Sol muscle (r = 0.76, P < 0.001), which decreases slightly as the age progresses in the wild-type mice (left graphs in Fig. 6, A and B). In contrast, in the mSod1\textsuperscript{G93A} mice, these parameters increase significantly with disease progression except for the current needed to generate maximum M-wave in Gs muscle (right graphs in Fig. 6, A and B).

Fig. 6. Changes in threshold current to elicit an M-wave and the current at half-maximum and maximum (max) M-wave in Gs and Sol in wild-type and in mSod1\textsuperscript{G93A}. A: threshold current, currents at half-maximum M-wave, and maximum M-wave in the Gs muscle do not change systematically from ages P48 to P162 in wild-type mice (open bars) and mSod1\textsuperscript{G93A} mice (closed bars) and mSod1\textsuperscript{G93A} mice (open bars) when they are younger (<P100) or older (>P100) than P100.

J Neurophysiol • doi:10.1152/jn.00507.2013 • www.jn.org
motor units with smaller axon diameters and therefore have higher activation thresholds (Kanning et al. 2010). The threshold current to elicit an M-wave in mSod1<sup>G93A</sup> mice when they are older than P100 in Gs and Sol is 1.53 and 2.09 times greater (respectively) than is required for the threshold current for the wild-types. In contrast, current at the half-maximal M-wave in mSod1<sup>G93A</sup> is 1.48 and 1.77 and maximal M-wave is 1.32 and 1.51 for Gs and Sol, respectively. The observation that the increase in threshold current is larger than the increase in current for half-max and maximal M-wave amplitude is in accordance with the notion that larger motor units (with lower activation thresholds) are lost preferentially during the ALS progression (Kanning et al. 2010).

Interestingly, the EMG activities of the Gs and Sol muscles during walking in mSod1<sup>G93A</sup> mice did not directly correlate to the muscle denervation or the M<sub>max</sub> amplitude (Fig. 7). Figure 7A illustrates three recordings obtained at three different ages in the same mSod1<sup>G93A</sup> mouse during walking (0.2 m/s) episodes that include three swing phases (indicated by the TA bursts). The EMG activity in Gs, however, being low at P70, increased considerably at P111 and was maintained until the P152. Finally, the EMG activity in VL was constant until P111 but dropped at P152. To illustrate the changes in the Sol muscle activity during walking, three recordings are shown in Fig. 7B from an individual mSod1<sup>G93A</sup> mouse at three different ages. Sol activity is first lower at P92 but increased to a comparable level at P124 and P145. The EMG activity was low when the recordings started P92 but increased gradually until P113 and stabilized. A more complete EMG pattern along with the angular joint movements in another mSod1<sup>G93A</sup> mouse is illustrated in Fig. 7C (same animal from which the M<sub>max</sub> data are presented as open circles in Fig. 5B). Notice that the Gs activity did not change as long as the M<sub>max</sub> remained at ~50% (P90–P103 and P131–P145) of its initial value at P70. However, when the M<sub>max</sub> dropped <50% (P110–P124 and after P152), the EMG activity also decreased drastically. Together, these data suggest that regardless of muscle denervation or the M<sub>max</sub>, as long as there are sufficient functional fibers, the nervous system is able to compensate for the loss to achieve the required amount of activity for behavior.

The main activity profile of the TA muscle in wild-type animals was one single burst during the first half of the swing phase regardless of the age of the animals (N = 4; Fig. 8A). In contrast, the TA activity profile changed from one solid burst during swing phase in younger ages to a “bursty” profile in all older mSod1<sup>G93A</sup> mice (N = 4; Fig. 8, B and C). This change in profile from solid to bursty occurred on average at P84.25 (±3.4 SD). Moreover, the bursty TA activity profile was accompanied by a “stuttered” leg movement during swing phase, which is depicted as the angular movement of the ankle joint to be jittery (Fig. 8, B and C). The stuttered swing movement, of the leg during walking, presumably caused by the bursty TA EMG profile, is the first symptom that can be detected by visual observation in the mSod1<sup>G93A</sup> mouse model.

**DISCUSSION**

The technique presented in this article allows continuing measurement of the M<sub>max</sub> also called the maximum compound muscle action potential (maxCMAP), and is a measure of the number of functional muscle fibers (Maathuis et al. 2013). The recordings can be performed for up to 3 mo in individual animals without any invasive procedures between recording sessions. The parameters measured on different days in wild-type mice show that the M-wave and EMG amplitudes during walking are largely constant in the wild-type animals. To demonstrate applicability of the technique in the context of degenerative diseases, I utilized the mSod1<sup>G93A</sup> mouse model of ALS. I demonstrated that in two out of three animals, the M<sub>max</sub>.
my data suggest that the MNs in mSod1G93A mice are less than the first recording performed on this animal. Furthermore, my data show that even though muscle denervation sessions, and presumably the initial drop occurred earlier (P70) from an individual mSod1G93A mouse (Mut1 in Fig. 5A). A close look into the wild-type graphs indicates that the 

measured in Gs started in similar $M_{\text{max}}$ values as in the wild-type animal but decreased in the following weeks when the animals were P53 and P77 and remained low throughout the recording sessions. In the remaining mSod1G93A mouse, the $M_{\text{max}}$ was at lower than wild-type values throughout the recording sessions, and presumably the initial drop occurred earlier (<P70) than the first recording performed on this animal. Furthermore, my data suggest that the MNs in mSod1G93A mice are less excitable than the wild-type MNs when they are older than P100. Additionally, my data show that even though muscle denervation occurs in mSod1G93A mice, the EMG activity pattern of muscles is regulated in a way that it does not change as the $M_{\text{max}}$ does when the animal walks. Differences in walking occur only when $M_{\text{max}}$ is decreased drastically.

The experiments described here are a combination of EMG recording and kinematic techniques, as previously described (Akay et al. 2006; Pearson et al. 2005), and tibial nerve stimulation. The cuff electrodes for the tibial nerve stimulation are a modified version of the cuff electrodes that were previously described (Carp et al. 2005). The modification of the cuff electrode and its fabrication allows decreasing the cuff diameter by ~35% and length by 66% compared with the cuff electrodes described by Carp et al. (2005). This decrease in cuff size is critical for the implantation of electrodes in young mice (P30) that weigh about 15–17 g. This is an important feature of this method that allows measurement of $M_{\text{max}}$ at presymptomatic ages. Moreover, the absolute value of the $M_{\text{max}}$ in all wild-type mice was in very similar ranges, making it possible to compare $M_{\text{max}}$ directly between individual mice.

In human experiments (Knikou 2008), it has been shown that H-wave amplitudes decrease when the M-wave amplitude increases and is abolished completely when the M-wave reaches its maximum. This is not observed in the present mice experiments (Fig. 2) where the H-wave after it reached a maximum decreased only slightly. Two possible explanations could account for this difference. First, not all MNs are activated by the electrical stimulation, meaning that there are always MNs left that are not in the refractory period to respond to the input from the primary afferents. Second, the H-waves recorded at higher stimulation intensities are mixed with the F-wave masking the abolition of the H-wave. The presented experiments do not allow differentiation between these possibilities, and it was not the aim of the present project, which only focused on the M-wave.

The presented method allows relatively stable $M_{\text{max}}$ measurements to be performed for several months without any additional surgeries or other invasive procedures in between recording sessions. This is a necessary feature, allowing use on mouse models of human disorders, such as spinal cord or brain injuries, or degenerative diseases, such as Parkinson’s disease, SMA, or ALS. In this study, mutant mice that model ALS, the mSod1G93A line (Gurney et al. 1994), serve as an example. A comparison of Figs. 3B and 5B illustrates that a decrease in $M_{\text{max}}$ in Gs (indicating muscle fiber denervation) and its changes over time in mSod1G93A mice can be detected, down to 1 mV, compared with wild-type mice in which $M_{\text{max}}$ in Gs varies between 12 and 28 mV. In contrast, the $M_{\text{max}}$ in Sol muscle remains relatively constant between 1.6 and 2.4 mV in the mSod1G93A mouse between P92 and P152 (Fig. 5C) compared with 2–4 mV from P77 to P162 in wild-type mouse (Fig. 3C). Previous histopathological observation suggests that slow contracting muscle fibers are more resistant to muscle denervation (Hegedus et al. 2008). The data presented here confirm that Sol muscle in mice is more resistant to denervation, measured as constant $M_{\text{max}}$ throughout the life of mSod1G93A mouse, suggesting that it consists mostly of slow contracting muscle fibers. In contrast, $M_{\text{max}}$ in Gs decreases between P45 and P84 and is reflected by time points of decreases (indicating reduced number of functional muscle fibers, suggesting net denervation) or increases (indicating a net increase in number of functional muscle fibers, suggesting net reinnervation by spared MN axons), similar to that previously described in human ALS patients (Maathuis et al. 2013).

A close look into the wild-type graphs indicates that the session-to-session changes in the average $M_{\text{max}}$ measurements
can be as high as the decreases or increases in the average $M_{\text{max}}$ measurements in the mSod1$^{G93A}$ mice. This complicates the interpretation of the data obtained from the mSod1$^{G93A}$ mice as to net denervation (decrease in $M_{\text{max}}$) or net reinnervation (increase in $M_{\text{max}}$). However, three observations suggest that the changes measured in mSod1$^{G93A}$ mice reflect net denervation or net reinnervation. First, the fluctuations in the wild-type animal are random with an overall increase of $M_{\text{max}}$ as the mouse ages from P47 to P140 (ANOVA: $P < 0.001$) for Gs and P77 to P162 (ANOVA: $P < 0.001$) for Sol. In contrast, the $M_{\text{max}}$ decreased over time in mSod1$^{G93A}$ animals (ANOVA: $P < 0.001$) in Gs and Sol. Second, the standard deviations of the $M_{\text{max}}$ measurements after denervation are much smaller (Fig. 5) than in the wild-type animals (Fig. 3), indicating variability within session is also decreasing, presumably due to decreasing number of motor units. Third, the amplitude of the EMG recordings during walking remains constant in wild-type (Fig. 4) animals but decreases and increases according to the changes in the $M_{\text{max}}$ measurements in the mSod1$^{G93A}$ mice (Fig. 7). Even though these observations indicate that the changes in $M_{\text{max}}$ in mSod1$^{G93A}$ reflect net denervation or net reinnervation, a definite clarification will require further attention in future projects.

In parallel to the $M_{\text{max}}$ recordings, the presented method also allows measurement of EMG activity in multiple muscles and kinematic parameters of leg movement during walking in a continuous manner. The data in Fig. 4 illustrate that, in wild-type mice after age P61, the angular movements of leg joints during stepping and the EMG activity pattern of Gs, VL, and TA muscles remain relatively stable. Younger mice have less EMG activity in Gs, presumably due to adolescent maturation of walking and/or weight gain; therefore, more Gs activity is needed to lift up the body.

Nevertheless, the long-term EMG recordings in parallel with the $M_{\text{max}}$ measurements suggest that loss of motor units to a certain degree is compensated in a way that animals can walk without difficulty or any overt sign of abnormality (Fig. 7). This type of compensation could be achieved through two mechanisms. First, weakness of particular muscles could be compensated by overactivation of synergist muscle that is not denervated, such as the Sol, through sensory feedback mechanisms (Pearson et al. 1999, 2003). Second, loss of motor units within individual muscles could be compensated by increasing the excitability of still-intact motor units. A candidate mechanism for the increase in MN excitability through cholinergic C-bouton modulation has been demonstrated (Miles et al. 2007; Wilson et al. 2004; Zagoraioi et al. 2009). The second possibility appears more likely, since no upregulation in Sol EMG activity during walking in aging mSod1$^{G93A}$ mice could be detected. However, further investigation is required to provide a definite answer, but the present data suggest that MNs of mSod1$^{G93A}$ mice are less excitable when the animals are older than P100 (Fig. 6).

The earliest behavioral abnormality of disease progression can be detected as a jittery ankle and stuttered leg movement during swing phase when the mSod1$^{G93A}$ mice walk (Fig. 8). On average, this abnormality appears around P84.25 ($\pm$3.4). The reason for this change could be due to changes in the premotor spinal circuitry that provide excitatory or inhibitory input to the MN activation, compensating for the motor unit loss (Avossa et al. 2006; Nagao et al. 1998; Schütz 2005).

Alternatively, it could be due to changes of intrinsic properties of the surviving MNs to compensate for the loss of motor units (Meehan et al. 2010; Quinlan et al. 2011; Zona et al. 2006). The data presented in this article suggest, regardless of whether it is a network or intrinsic compensation, it occurs around P84. Therefore, it would be very informative to measure and compare intrinsic MN properties of mSod1$^{G93A}$ mice before and after P84.

In conclusion, the method presented in this paper is very suitable for tracking changes in the spinal sensory motor circuitry for over 3 mo, and therefore it is very valuable for neuroscience research aimed at understanding the progression of degenerative diseases or the effect of injuries. Furthermore, it can be used to measure effects of potential therapeutic approaches. The $M_{\text{max}}$ measurements from Gs and Sol of ALS model mice suggest that denervation process can effectively be measured for 3–4 mo. This is an important feature of the technique because the mSod1$^{G93A}$ mice have a life span of ~160 days. The data with mSod1$^{G93A}$ suggest that as the muscle denervation progresses, massive compensation occurs within the spinal cord due to changes of intrinsic MN properties or spinal premotor network function, offsetting the loss of motor units. However, around P84, the first sign during walking is detected as abnormal TA activity profiles resulting in anomalous swing movement.

ACKNOWLEDGMENTS

I thank Anatoly Voskresenskiy and FangHua Li for their technical assistance and Olga Akay for drawing the schematic of the cuff electrode that is illustrated in Fig. 1B. I am grateful to Olga Akay and Drs. Andrew Murray and Angsa Büschges for their comments on the manuscript.

GRANTS

This work was supported by the Department of Neuroscience, Department of Neurosurgery, and the Center for Motor Neuron Biology and Disease of the Columbia University.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

T.A. conception and design of research; T.A. performed experiments; T.A. analyzed data; T.A. interpreted results of experiments; T.A. prepared figures; T.A. drafted manuscript; T.A. edited and revised manuscript; T.A. approved final version of manuscript.

REFERENCES


