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

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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 mSod1<sup>G93A</sup> mice, which model human amyotrophic lateral sclerosis (ALS). The denervation process of gastrocnemius and soleus muscles in mSod1<sup>G93A</sup> 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 tibialis anterior activity profile that is manifested in subtle but 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 diseases, 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 (mSod1<sup>G93A</sup>) 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 (Quinlan 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 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 mSod1<sup>G93A</sup> (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 (M<sub>max</sub>) 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, mSod1<sup>G93A</sup>, 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...
the Care and Use of Laboratory Animals and approved by the
Institutional Animal Care and Use Committee at Columbia University.

Animal model. To characterize the muscle denervation and changes
in locomotor behavior in individual mice that mimic ALS, a trans-
genetic mouse line expressing the mutant form of Cu,Zn superoxide
dismutase 1 enzyme (substitution of Gly93 with Ala; mSod1<sub>G93A</sub>)
was used. In total, five wild-type mice and five mSod1<sub>G93A</sub> mice (low
expressor; Gurney et al. 1994) were used for this study. It has been
shown previously that the mSod1<sub>G93A</sub> mice develop stereotyped
phenotypes resembling the symptoms in ALS patients. The pheno-
types 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 record-
ing 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 AlliedSilicone
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-

Fig. 1. Fabrication of the nerve stimulation electrode. A: a
cross-section of a nerve cuff electrode during its fabrication is
illustrated from left to right. Bared regions of 2 wires are
driven through a silicone tube with help of a 27-gauge needle
and a 6-0 suture so they are all parallel. The bared wires and
the suture are evenly covered with silicone adhesive. Finally, the
tube is cut lengthwise as shown at the very right. B: schematic
presentation of the cuff and the description of all its components.
C: picture of 1 finished nerve cuff electrode. D: picture of a
ready-to-implant set of electrodes. EMG, electromyographic.
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 Gs, 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 (analgesic) was injected subcutaneously while the incisions on the legs were closed, and the headpiece was stitched to the cuff were sutured on the surface of the surrounding fascia. The muscles that were recorded in these experiments were the vastus lateralis (VL), lateral Gs, 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 (analgesic) 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-dimensiona1 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 anesthesi a, 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 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 McDougal 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 spinoally 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 (~<100 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 contrast, reached maximal amplitude followed by a slight decrease with the increasing stimulation strength. Two observations were different from previous observations on H-reflex experiments performed on humans (Knikou 2008). First, current threshold was not consistently lower for the H-wave than for the M-wave as has been observed in humans. On the 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. 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: $P < 0.001$; Fig. 3C). 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. Therefore, in the case where the number of activatable muscle fibers would decrease (for example, due to denervation), the $M_{\text{max}}$ would also decrease. In contrast, if the number of activatable muscle fibers increased (for example, due to reinnervation), the $M_{\text{max}}$ would also increase. Since denervation and innervation in ALS presumably occur simultaneously, the decrease in $M_{\text{max}}$ would indicate net denervation, whereas increase would indicate net reinnervation.

Another important feature of the method is that it allows the measurement of EMG activity along with angular joint movements of the leg joints during freely walking mice in parallel with $M_{\text{max}}$ recordings for several months (Fig. 4). Figure 4A illustrates EMG recordings from TA, Gs, and Sol muscles...
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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 ($\pm$SD, $n = 50$) $M_{\text{max}}$es 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 ($\pm$SD) of $M_{\text{max}}$ recorded from all wild-type
animals at all days. WT1–5 indicate the number of specific wild-type animal.
Notice that the gray pentagon in B and C illustrates data from the same
wild-type animal (WT3). P, postnatal day.

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 ac-
tivity 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 P53, and EMG recordings
from Gs, VL, and TA synchronously with kinematic measure-
ments were started at age P48 and continued until the mouse
was P115. Overall, the EMG patterns and the angular joint
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,
mSod1$^{G93A}$. 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 P151, 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:

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
mouse (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. deg, Degrees.
with wild-type values. In contrast, the M_max values measured for Mut3 and averages for Mut1, although significantly smaller (average amplitude of the first (for Mut3) and the first two
manner with timing of events variable from animal to animal. but the recordings were obtained from Sol muscle of 2 mSod1G93A animals.

Fig. 5. Recordings of M_max for mSod1G93A mice. A: 5 average M_max EMG responses (n = 50) to 400 μA at 5 different ages in the same mSod1G93A animal. Note that instead of a slight increase as in the wild-type mice (Fig. 3), the M_max is considerably smaller at P124, partly recovered at P131, and dropped again at P152. B: graph illustrating average (±SD) M_maxes recorded in Gs muscles in 1-wk intervals from 2 different mSod1G93A animals illustrating that changes in M_max over time in mSod1G93A mice decrease in stepwise manner with timing of events variable from animal to animal. C: same as in B, but the recordings were obtained from Sol muscle of 2 mSod1G93A animals. The M_max increases gradually until P90 but then stays constant as the disease progresses. The diamonds on the very left in B and C indicate mean (±SD) of the M_max values measured in wild-types presented in Fig. 3, B and C, respectively. The horizontal solid and dotted lines, respectively, indicate the wild-type mean and SD values. Mut1–5 indicate the number of specific mSod1G93A animals.

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_max values obtained from mSod1G93A mice were significantly lower than the average M_max values measured from all wild-type mice for Gs and Sol but all 1-day measurement for Sol. In two out of three M_max measurements in Gs in mSod1G93A 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_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 mSod1G93A 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 mSod1G93A 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 mSod1G93A 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). Furthermore, comparison of the threshold current and current for half-maximum and maximum M-wave shows no difference between wild-types and mSod1G93A when the mSod1G93A mice are younger than P100 but are significantly higher when they are older than P100 (Fig. 6C). This indicates that when the mSod1G93A 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
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

**Fig. 7. Recordings of the locomotor pattern and angular joint movements for ~3 mo in mSod1<sup>G93A</sup> mice.** A: EMG activities of the TA, Gs, and VL 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 mSod1<sup>G93A</sup> mouse (Mut2 in Fig. 5B). The recordings are from the same animal where M<sub>max</sub> is presented in Fig. 5B (gray circles). Note that Gs activity at P70 in parallel with M<sub>max</sub> is low at P70 and larger at P111 and P152 when the M<sub>max</sub> is partly recovered, a trend similar to the M<sub>max</sub> amplitudes. B: EMG activities of the TA and Sol muscles during a walking sequence at 0.2 m/s that includes 4 swing phases (indicated by TA activity) at 3 different ages from an individual mSod1<sup>G93A</sup> mouse (Mut5 in Fig. 5C). Sol activity profile is lower at P92 but increases later. C: 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. The averages are triggered around the beginning of a stance phase (vertical lines) at 12 ages of the same mSod1<sup>G93A</sup> mouse. The data are from the same animal as in Fig. 5B (Mut1). Note that reduction of M<sub>max</sub> by ~50% does not cause changes in activity profile of Gs during walking. Only when M<sub>max</sub> drops <50% (P110, P124, and P152) is the EMG activity profile of Gs drastically reduced during walking.

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 excitable than the wild-type MNs when they are older than P100. Furthermore, when the animal walks, the EMG activity pattern of muscles occurs in mSod1G93A mice, the EMG activity pattern of muscles 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; Zagoraiou 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 (±3.4). The reason for this change could be due to changes in the premotor spinal circuitry that provide excitationary 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.

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DISCLOSURES

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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.

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