Neuromuscular transmission failure and muscle fatigue in ankle muscles of the adult rat after spinal cord injury

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ANATOMIC INSTABILITIES have been demonstrated in specific populations of hindlimb neuromuscular junctions (NMJ) in adult rats after transection of the thoracic spinal cord (3). Changes include the withdrawal of presynaptic terminal axons, loss of postsynaptic ACh receptors, and reduced apposition of pre- and postsynaptic components and terminal sprouting. In addition, spontaneous potentials (fibrillation potentials and positive sharp waves) were also observed in muscles innervated by spinal cord segments distal to the level of direct injury in these spinal cord injured animals (4). Fibrillations are a hallmark of muscle denervation, and their presence combined with the observed anatomic changes suggest that NMJ transmission may be compromised. Studies on the diaphragm of rats with the observed anatomic changes suggest that NMJ transmission failure for the TA between control and spinal animals. These results demonstrate that, although there may be a mild decrement in NMJ function, NMJ transmission remains largely intact for supramaximal nerve stimulation.

To determine whether these localized anatomic alterations compromise NMJ function, we electrophysiologically assayed synaptic function at NMJs after experimental spinal cord injury (SCI). Specifically, we determined the contribution of neuromuscular transmission failure to ankle flexor or extensor muscle fatigue using repetitive stimulations (via nerve and muscle) in control and spinalized animals. We specifically chose to study whole muscle force output as we were interested in the potential functional deficits brought upon by the observed structural changes in NMJs. To our knowledge, there have been no prior in situ animal studies of hindlimb muscles NMJ failure in response to repetitive stimulation.

MATERIALS AND METHODS

Animal groups. Thirty-one adult female Sprague-Dawley rats, weighing 250–300 g, were used in this study. Female rats were used to facilitate bladder management posttransection. The contribution of neuromuscular transmission failure to muscle fatigue was studied in two hindlimb muscles: the medial gastrocnemius (MG) and tibialis anterior (TA). Animals were divided into five groups: MG force measurements in intact control animals (MG/Ctrl; 6 rats), MG measurements 2 wk after SCI (MG/SCI-2; 8 rats), TA measurements in intact animals (TA/Ctrl; 7 rats), TA measurements 2 wk after SCI (TA/SCI-2; 6 rats), and TA measurements 6 wk after SCI (TA/SCI-6; 4 rats). All experiments were performed at Drexel University College of Medicine (Philadelphia, PA). The experimental protocol was approved by Drexel University’s Institutional Animal Care and Use Committee, and animal care followed National Institutes of Health guidelines.

Spinal transection. The spinal transection procedure has been previously described (4). Briefly, rats were deeply anesthetized with an intraperitoneal injection of ketamine (80 mg/kg), xylazine (8 mg/kg), and acepromazine (0.6 mg/kg). The adequacy of anesthesia was confirmed by the absence of corneal reflexes and response to paw pinch. Under aseptic conditions, the spinal cord was exposed by a partial laminectomy of the ninth thoracic vertebra (T9). After the topical application of lidocaine hydrochloride (1%, 2–3 drops), a 2 mm length of cord was removed en bloc. The resulting cavity was filled with an adipose tissue graft from the dorsal subcutaneous pad. The overlying muscles and fascia were closed in layers using sutures, and the skin incision was closed with wound clips. After surgery, bladders were manually expressed three times daily for ~2 wk, until reflex voiding returned. Ampicillin was administered to prevent infection. Specialized bedding was also provided to prevent pressure ulceration or other complications.

Muscle/nerve fatigue measurement experimental procedures. Animals were anesthetized as described above. Either the MG or TA muscle was exposed and mobilized from adjacent muscles while preserving the blood and nerve supplies using blunt dissection. The accompanying nerve was then identified, and branches to the neighboring muscles were cut or crushed to prevent the activation of neighboring muscles and possible contamination of the force measurements. The distal tendon was freed and sutured to a metal clip, after which two insulated fine wire electrodes (catalog no. 793400, A-M Systems, Carlsborg, WA) with exposed stainless steel tips were inserted into the muscle belly using a 23-gauge hypodermic needle. The anesthetized animal was then transferred to the electrophysiology stage where the freed tendon, via a clip, was attached to a force transducer (Grass Technologies FT03E, West Warwick, RI).

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the incised skin as flaps, muscles and nerves were immersed in a mineral oil bath to prevent desiccation and maintain tissue viability. The nerve of interest was then placed onto a bipolar hook. The MG muscle was activated by stimulating the tibial nerve, and the TA muscle was activated by stimulating the peroneal nerve (see Fig. 1). Nerves were stimulated using 0.1-ms pulses at supramaximal current intensity (2 mA) delivered at 40 Hz in 330-ms duration trains. Muscles were stimulated using supramaximal pulses (10 mA) of 1-ms duration at 40 Hz in 330-ms trains. The repetitive stimulation parameters were selected to match the ones in previous studies (2, 7, 10) evaluating muscle fatigue or NMJ failure. We attempted to study both legs in all animals, although technical challenges associated with the preparation precluded this in some animals.

The optimal length was identified by applying short trains of direct muscle stimulation and adjusting muscle length until maximal isometric force was obtained. To determine the ratio of maximum force obtained with nerve versus direct muscle stimulation, three maximal direct muscle stimulations were performed separated by 2-min rest periods. This was followed by three maximal nerve stimulations performed in similar fashion. After a 5-min rest period, repetitive stimulation protocols were initiated to determine fatigue and NMJ transmission failure as in Refs. 7 and 8. Repetitive stimulation of the stimuli trains described above (40 Hz, 330-ms duration) was delivered, via the nerve, at 1-s intervals for a 2-min period. Superimposed direct muscle stimulation (also as described above) was performed every 15 s (Fig. 1). Three trials were performed for each muscle and separated by 5-min rest intervals to allow for muscle recovery. Stimuli were delivered via two isolated pulse stimulators (model 2100, A-M Systems) controlled using a programmable pulse generator (Master-8, AMPI, Jerusalem, Israel). The evoked isometric force responses were displayed on an oscilloscope and sampled at 1,000 Hz on a computer. Data acquisition was controlled by customized software (Labview, National Instruments, Austin, Texas) running on a personal computer.

At the conclusion of the experiment, animals were euthanized with pentobarbital sodium (150 mg/kg) and perfused through the heart with 4% paraformaldehyde. The spinal cords of approximately one-third of the animals were dissected out and inspected. The method of transection, removal of 2 mm of the spinal cord en bloc, produced a readily apparent defect in all dissected animals. In our prior report (4) using the same method of transection, we have confirmed the completeness of our lesions using Nissl/myelin stain and immunohistochemistry with antibody raised against neurofilament. In every case, we observed an absence of spinal cord tissue at the surgical site with Nissl/myelin stain and the absence of axons with immunohistochemistry for neurofilament.

**Data analysis.** Fatigue and the extent of transmission failure at NMJ synapses were determined based on the difference in force generated with nerve versus direct muscle stimulation, as described by Ref. 7. By comparing the forces obtained through direct activation of the muscle fibers (muscle stimulation) with the ones obtained by activation through the NMJ (nerve stimulation), we obtain a measure of the failure rate at the NMJ under repetitive stimulation. To normalize the evoked force response across observations, the ratio of maximum force after 2 min of stimulation over the maximum “initial” force at 15 s was determined for each type of stimulation (muscle and nerve). Since potentiation was often observed at the beginning of the fatigue tests (see Fig. 1), we decided to use the 15-s point as the force reference for determining the magnitude of transmission failure at the conclusion of the 2-min runs.

The relative contribution of NMJ failure to force reduction with repeated stimulation was estimated using the following equation:

\[
\text{Transmission failure} = \frac{(F - MF)/(1 - MF)}{F} \tag{1}
\]

where F is the percent decrement in force during repetitive nerve stimulation and MF is the percent force decrement during direct muscle stimulation. The equation provides a measure of NMJ junction failure since it evaluates the difference between the force decrement over repetitive stimuli when activating the muscle via the NMJ (nerve stimulation) alone and through direct depolarization of the muscle fibers (muscle stimulation superimposed over nerve stimulation). Because there were no differences between values for the neuromuscular transmission failure from the three trials on each leg (one-way analysis of variance, repeated measures).
repeated-measures ANOVA, Wilk’s $\lambda = 0.97$, $F_{2,35} = 0.60$, $P = 0.555$), data from the three trials were averaged together for further analysis. Differences in the percentage of force decrement and in neuromuscular transmission failure between groups were examined using the Mann-Whitney nonparametric test and repeated-measures ANOVA (time $\times$ group). The Wilcoxon nonparametric test was used to analyze the differences in force between muscle and nerve stimulation in each group. Statistical significance was established at the 0.05 level. All experimental data are presented as means $\pm$ SE.

RESULTS

Muscle force output for single train stimuli. The similarity in maximal isometric force obtained with nerve stimulation compared with direct muscle stimulation was calculated from the average of three trials using supramaximal pulses, as described above. The average ($\pm$SE) force output from nerve stimulation compared with direct muscle stimulation was as follows for the various groups: 91.1 $\pm$ 3.6% for MG/Ctrl, 83.2 $\pm$ 4.5% for MG/SCI-2, 91.5 $\pm$ 2.0% for TA/Ctrl, 86.5 $\pm$ 2.4% for TA/SCI-2, and 94.5 $\pm$ 1.8% for TA/SCI-6. No significant differences between groups were observed for this measurement (one-way ANOVA, $P = 0.154$). Maximal contraction for each group was 5.20 $\pm$ 0.53 N for MG/Ctrl, 3.15 $\pm$ 0.39 N for MG/SCI-2, 5.35 $\pm$ 0.57 N for TA/Ctrl, 3.99 $\pm$ 0.38 N for TA/SCI-2, and 5.07 $\pm$ 0.39 N for TA/SCI-6. The decrease in maximal force 2 wk after SCI was statistically significant for both muscles (one-way ANOVA, $P = 0.002$; least significant difference post hoc tests: $P = 0.003$ for the MG muscle and $P = 0.037$ for the TA muscle). In summary, initial force output was similar for nerve or muscle stimulation and muscle force decreased 2 wk post-SCI.

Muscle force output for multi train stimuli. The decrease in the percentage of initial force after 2 min of repetitive stimulation was statistically significant ($P < 0.001$ for each group) for both direct muscle and nerve stimulation. Within animals, the initial forces were comparable for nerve and direct muscle stimulation. As the repetitive stimulation progressed, differences between the force generated with nerve and direct muscle stimulation became apparent and significant from 30 s onward ($P < 0.001$ for each group; Fig. 2). The forces at the conclusion of the 2-min stimulation period, as a percentage of initial force, are shown in Table 1 for each group. With stimulation via the nerve, no significant differences in forces were observed between control and spinalized animals at any time point. For direct muscle stimulation, significant differences were observed at times of 30 and 45 s ($P = 0.007$ and 0.044, respectively; Fig. 2) between TA/Ctrl and TA/SCI-2 only.

The contribution of NMJ transmission failure to muscle fatigue after 2 min of repetitive nerve stimulation is shown in Fig. 3. Values for NMJ transmission failure (Eq. 1) for each group at 120 s are shown in Table 1. Neuromuscular transmission failure was consistently larger in spinalized rats than in controls. A trend was found for the TA/SCI-2 group compared with the TA/Ctrl group with $P = 0.087$ at 90 s, $P = 0.063$ at 105 s, and $P = 0.053$ at 120 s (Fig. 3). Further analysis of the animals in which we were able to conduct all three 2-min trials revealed a significant difference ($P = 0.047$) between the TA/SCI-6 and TA/Ctrl groups using a double-nested repeated-measures ANOVA (with trial and time as levels). No other significant differences were found.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Muscle Stimulation, %initial value</th>
<th>Nerve Stimulation, %initial value</th>
<th>Neuromuscular Junction Transmission Failure, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medial gastrocnemius muscle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>56.6 $\pm$ 3.8</td>
<td>34.2 $\pm$ 3.7</td>
<td>38.5 $\pm$ 7.2</td>
</tr>
<tr>
<td>2 wk post-SCI</td>
<td>14</td>
<td>59.5 $\pm$ 2.5</td>
<td>28.0 $\pm$ 3.9</td>
<td>52.8 $\pm$ 5.4</td>
</tr>
<tr>
<td>Tibialis anterior muscle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>57.0 $\pm$ 5.3</td>
<td>39.2 $\pm$ 2.7</td>
<td>29.0 $\pm$ 3.6</td>
</tr>
<tr>
<td>2 wk post-SCI</td>
<td>11</td>
<td>57.0 $\pm$ 3.0</td>
<td>32.1 $\pm$ 4.4</td>
<td>45.1 $\pm$ 6.5</td>
</tr>
<tr>
<td>6 wk post-SCI</td>
<td>7</td>
<td>54.6 $\pm$ 4.4</td>
<td>32.4 $\pm$ 6.5</td>
<td>43.9 $\pm$ 9.5</td>
</tr>
</tbody>
</table>

Values are means $\pm$ SE; n, no. of legs. SCI, spinal cord injury.
and colleagues (8) observed a reduction in the diaphragm NMJ failure after hemisection and an increase with inactivity induced by TTX. The spinal model may fall between those other two models of muscular inactivity.

As measured by the progressive decrement in force generated with each stimulus train, muscles fatigued when stimulated either via the nerve or directly through the muscle, with the fatigue rate being greater with nerve stimulation. The rates of fatigue and transmission failure were comparable for the two predominantly fast-twitch muscles studied (5, 9). With nerve stimulation, the extent of fatigue appeared to be slightly greater in SCI animals than in controls (Fig. 2). Although these differences were not significant, dysfunction at the NMJ level could still be responsible since fatigue at 120 s was essentially the same between control and SCI animals with direct muscle stimulation.

A previous study (3) has demonstrated anatomic instability of NMJs in ankle flexors caudal to SCI, with the alterations being more pronounced in the TA than MG muscle. Based on the results of the present study, it appears that post-SCI anatomic changes do not induce significant NMJ transmission failure when the nerve is stimulated at a supramaximal level. It is interesting to note, however, that statistically significant differences in NMJ failure were only found for the muscle showing the greatest anatomic changes. Mantilla and colleagues (6) observed an increase in synaptic vesicle pool size and density after spinal hemisection, an increase that may explain the reduction in NMJ failure in that population. Our higher NMJ failure in SCI rats would suggests that a similar phenomenon did not occur in completely transected animals or that the benefits in synaptic efficacy were offset by the morphological changes occurring at the NMJ.

Our results do not exclude a role for NMJ modifications in the occurrence of spontaneous potentials after human and experimental SCI. Indeed, a small number of disorganized synapses could cause fibrillation potentials without impacting the force generated by the whole muscle during nerve stimulation.

A larger question is whether this study provides us with clues about the functional state of NMJs after human SCI. Based on our study’s results, NMJ transmission failure may be happening after SCI; however, the extent in humans would likely be mild if it is analogous to what we observed in the adult rat. One important caveat is that the longest duration studied was 6 wk, whereas humans can live years and even decades after severe SCI. The long-term ramifications for peripheral nervous system and NMJ function could be quite different. In addition, it is known that human muscles show an increased predisposition to fatigue, particularly with functional electrical stimulation, after SCI. While this has been largely attributed to type I to type II fiber conversion, NMJ function could also play a role. Future studies are needed to answer these questions.

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