Corticosteroid effects on diaphragm neuromuscular junctions

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Sieck, Gary C., Roland H. H. Van Balkom, Y. S. Prakash, Wen-Zhi Zhan, and P. N. Richard Dekhuijzen. Corticosteroid effects on diaphragm neuromuscular junctions. J. Appl. Physiol. 86(1): 114–122, 1999.—The effects of corticosteroid (CS) treatment (prednisolone continuously administered subcutaneously at a flow rate of 2.5 µl/h, daily dose 5.6 mg/kg, for 3 wk) on neuromuscular junction (NMJ) morphology and neuromuscular transmission in rat diaphragm muscle (Di mus) were compared with weight-matched (Sham) and ad libitum fed control (Ctl) groups. Fibers were classified on the basis of myosin heavy chain (MHC) isoform expression. CS treatment caused significant atrophy of fibers expressing MHC2x (type IIx), either alone or with MHC2b (type IIx/b). Fibers expressing MHC slow (type I) and MHC2a (type IIa) were unaffected by CS. The planar areas of nerve terminals and motor endplates at type IIx/b fibers were smaller in CS-treated Di mus compared with Sham and Ctl. However, CS-induced atrophy of type IIx/b fibers exceeded changes in NMJ morphology. Thus, when normalized for fiber diameter, NMJs were relatively larger in the CS-treated group compared with Ctl. Neuromuscular transmission failure, assessed in vitro by comparing force loss during repetitive (40 Hz) nerve vs. direct muscle stimulation, was less in CS-treated Di mus. These results indicate that alterations in NMJ morphology after CS treatment are dependent on fiber type and may contribute to improved neuromuscular transmission.

EXOGENOUSLY ADMINISTERED corticosteroids (CS) are used clinically to treat a variety of pulmonary conditions, including asthma and severe chronic obstructive pulmonary disease. Several studies have reported atrophy of diaphragm muscle (Di mus) fibers and also muscle weakness (24, 45, 47). In the Di mus, the effects of CS treatment, especially in high doses, appear to be manifested predominantly at type IIx/b fibers (24, 45, 47).

Several previous studies have reported that CS treatment induces morphological changes at the neuromuscular junction (NMJ) (7, 8, 22, 23, 44), but only a few studies (7, 8) have examined fiber type differences in CS-induced changes in NMJ morphology. In these studies, which were performed in limb muscles predominantly expressing a single fiber type, NMJ adaptations were found to be more prominent at type I fibers. The purposes of the present study were to examine the effects of 3 wk of prednisolone treatment on the morphological properties of pre- and postsynaptic elements of the NMJ on type-identified fibers of the rat Di mus and to evaluate Di mus neuromuscular transmission. We hypothesized that the effects of prednisolone would be more pronounced on the NMJs of type IIx/b fibers.
METHODS

Twenty-four male Sprague-Dawley rats were randomly divided into three groups: 1) normal controls (Ctl; n = 8); 2) surgical sham and weight-matched controls (Sham; n = 8); and 3) CS-treated (CS; n = 8). The animals were housed in separate cages under a 12:12-h light-dark cycle, fed with Purina Rat Chow, and provided with water ad libitum. Animals in the Ctl and CS groups were also provided food ad libitum, whereas the rats in the Sham group were given limited quantities of food to match their weight-growth curve with that of the CS group. Body weights were measured every 3 days.

After 3 wk, animals were anesthetized with pentobarbital sodium (70 mg/kg), and the DImus was rapidly excised. Five muscle segments (2–3 mm wide) were dissected from the midcostal region of the right and left sides of the DImus. In one muscle strip from the right side, neuromuscular transmission failure was assessed by repetitive stimulation of the phrenic nerve (see Assessment of neuromuscular transmission failure). In the remaining strips, resting (excised) muscle length was measured by using digital calipers, and the strip was then stretched to 1.5 times this excised length, to approximate optimal length for force production (30), before the strip was pinned to a Sylgard-lined petri dish for immunocytochemical analysis.

All procedures were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic, and procedures were in strict accordance with the American Physiological Society Animal Care Guidelines. Surgical procedures were performed under aseptic conditions. The recovery of animals from surgery was carefully monitored.

CS treatment. Surgical procedures were performed only on animals in the CS and Sham groups. Under ketamine (60 mg/kg) and xylazine (2 mg/kg) anesthesia, a miniosmotic pump (Alzet 2ML4) filled with either 37.5 mg/ml prednisolone sodium succinate (Upjohn) in aqueous suspension (CS group) or sterile physiological saline (Sham group) was implanted subcutaneously in the dorsum of each animal. The miniosmotic pump provided a continuous flow rate of 2.5 µl/h; therefore, the daily prednisolone dose was 5.6 mg/kg. This dosing was primarily selected to match previous studies (4, 42–44) and was not meant to be a physiological replacement dose. At the end of a 3-wk treatment period, the remaining amount of solution in the pump was measured to ensure adequate drug delivery. In addition, at the time of the terminal experiment, 3 ml of blood was collected to measure both serum prednisolone (18) and thyroid hormone [3,3',5'-triiodo-L-thyronine (T3) and thyroxine (T4)] levels (15).

Immunohistochemistry. Detailed descriptions of the three-color fluorescent immunohistochemical technique used to label nerve terminals, motor endplates, and muscle fibers have been published recently (32). Briefly, motor endplates were first labeled by incubation of each muscle strip in 5–10 µg/ml tetramethylrhodamine ✶-bungarotoxin (Molecular Probes) in phosphate buffer. The samples were then washed and immersion fixed in 2% paraformaldehyde. The fixed samples were blocked for nonspecific staining by using 4% normal donkey serum (0.15 M NaCl) containing 0.3% Triton X-100. The tissue was then incubated in a primary mixture of 1:200 donkey antiprotein gene product (Biogenesis), to label axons and nerve terminals, and any one of the following four antibodies specific to different MHC isoforms: 1) 1:400 mouse anti-MHC all2X IgG (Novocastra); 2) 1:200 mouse anti-MHC2A IgG (16); 3) 1:200 mouse anti-MHC2B IgM (37); or 4) 1:20 mouse anti-MHC2X IgG (Jackson Immunoresearch) and 1:200 Cy5-conjugated donkey antimer IgG or IgM (Jackson Immunoresearch). All samples were finally washed, blotted dry, mounted on slides, and coverslipped with low-fluorescence immersion oil (refractive index 1.515; Cargille Laboratories).

After incubation in these primary antibodies, the samples were washed and incubated further in a secondary cocktail of 1:100 fluorescein-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch) and 1:200 Cy5-conjugated donkey antimer IgG or IgM (Jackson Immunoresearch). All samples were finally washed, blotted dry, mounted on slides, and coverslipped with low-fluorescence immersion oil (refractive index 1.515; Cargille Laboratories).

Confocal imaging and analysis. Detailed descriptions of the three-color confocal imaging and analysis procedures have also been recently published (32). Briefly, optical sections of labeled NMJs and muscle fibers were obtained by using a Bio-Rad MRC500 confocal system mounted on an Olympus BH2 microscope and equipped with an Ar-Kr laser and a z-axis focus controller. A ×40 1.25 numerical aperture oil-immersion objective lens was used, and the step size for optical sectioning was set to 0.8 µm, matching the optical section thickness for the confocal system (34). Morphometric and registration validations of the confocal imaging technique were performed by using multicolor fluorescent latex beads, as described previously (32). Two-dimensional (2-D) projection images were obtained by superimposing a stack of optical sections.

A comprehensive image-analysis software package (ANALYZE, Biomedical Imaging Resource, Mayo Foundation) running on a Sun 4/330 UNIX workstation was used to create three-dimensional (3-D) reconstructions of the digitized images and to make 2-D and 3-D morphometric measurements. Gray scale images were converted to binary images by using an intensity threshold. An automated procedure was used to delineate the borders of nerve terminals and endplates. Planar areas of nerve terminals and endplates were measured from the 2-D projections and normalized to muscle fiber diameter. The extent of overlap between nerve terminal and endplate was estimated by subtracting the binary image of the nerve terminal from the binary image of the endplate. The pattern of arborization of nerve terminals and endplates was quantified from optical sections by using a tree-tracing tool in ANALYZE. Details of this analysis technique have been previously published (32), and a schematic representation of the procedure is shown in Fig. 1. The point of origin for each nerve terminal was defined as the first branch point of the axon. The longest uninterrupted branches were classified as primary branches, and daughter branches arising from the primary branches, regardless of thickness, were classified as secondary branches. Branch length was measured along the center of the branch. The total number of branches and total branch length were determined. The average distance between the occurrence of secondary branches along a primary branch (individual branch length) was used as an index of arborization.

![Image](https://via.placeholder.com/150)

Fig. 1. Schematic of procedure used to characterize branching patterns of nerve terminals and motor endplates. Origin of neuromuscular junction was defined as the first point of branching. Primary branches were defined as the longest segments, secondary branches as those emanating from primary branches, and so on.
Assessment of neuromuscular transmission failure. The procedures for assessing neuromuscular transmission failure in the rat Di\textsubscript{mus} have been described previously (13, 17). A muscle strip from the right midcostal region, together with a 1- to 2-cm length of phrenic nerve, was mounted vertically in a glass chamber that contained oxygenated (95% O\textsubscript{2}-5% CO\textsubscript{2}) Ringer solution (in mM: 137 Na\textsuperscript{+}, 5 K\textsuperscript{+}, 5.04 Ca\textsuperscript{2+}, 2 Mg\textsuperscript{2+}, 121 Cl\textsuperscript{−}, 20 HCO\textsubscript{3}\textsuperscript{−}, and 1.9 HPO\textsubscript{4}\textsuperscript{2−}; pH 7.4) that was maintained at 26°C. The muscle was attached at one end to a calibrated force transducer and at the other end to a micromanipulator for adjustment of muscle length. The muscle was stimulated directly (1-ms pulses), through platinum-plate electrodes placed on either side of the muscle, by using a Grass stimulator and a power amplifier (Section of Engineering, Mayo Clinic). Muscle fiber length was incrementally adjusted until maximal isometric twitch responses were obtained (optimal length). The phrenic nerve was stimulated through a suction electrode by using 0.2-ms duration pulses. In both cases, stimulus intensity was increased until maximal twitch-force responses were obtained, and then intensity was set at 125% of this value (supramaximal intensity).

The method used to estimate the relative contribution of neuromuscular transmission failure to total Di\textsubscript{mus} fatigue has been previously described in detail (1, 17, 21, 31). Briefly, the phrenic nerve was stimulated repetitively at 40 Hz in 330-ms-duration trains repeated every second (duty cycle 33%) for a 2-min period. Direct muscle stimulation was superimposed on nerve stimulation to estimate the extent of neuromuscular transmission failure, using the formula

\[
\%\text{Neuromuscular transmission failure} = (\Delta N - \Delta M)/(1 - \Delta M)
\]

where \(\Delta N\) is force loss during nerve stimulation (normalized to initial muscle force) and \(\Delta M\) is force loss during direct muscle stimulation (normalized to initial muscle force).

Statistical analysis. Muscle fibers were classified on the basis of innervation density for the different MHC antibodies. Fibers classified as type I were innervated only by the anti-MHC\textsubscript{slow} antibody. Similarly, type I\textsuperscript{a} fibers were reactive only for the anti-MHC\textsubscript{C2A} antibody. Approximately 22% of all Di\textsubscript{mus} fibers were not innervated for the anti-MHC\textsubscript{C1\textsubscript{HIX}} antibody, and they were thus classified as type IIx. Approximately 12% of all Di\textsubscript{mus} fibers were innervated for the anti-MHC\textsubscript{C2B} antibody, but previously we found that most of these fibers also expressed the MHC\textsubscript{C2X} isoform (39). Given the relatively rare incidence of “true” type I\textsuperscript{b} fibers in the rat Di\textsubscript{mus} (4-8% (39)), and the fact that it was not possible to distinguish these fibers from those co-expressing the MHC\textsubscript{C2X} isoform by immunohistochromy, we combined the fibers expressing the MHC\textsubscript{C2X} and MHC\textsubscript{C2B} isoforms into a single group classified as type II\textsuperscript{bx}.

RESULTS

Prednisolone and thyroid hormone levels. Serum prednisolone levels were below the detectable range (<0.1 µg/dl) in Ctl and Sham groups, whereas in CS-treated animals, prednisolone levels were 4.9 ± 1.0 µg/dl. Serum T\textsubscript{3} and T\textsubscript{4} levels were not significantly affected by prednisolone treatment (Ctl: T\textsubscript{3} 46 ± 3 ng/dl, T\textsubscript{4} 4.0 ± 0.2 µg/dl; Sham: T\textsubscript{3} 48 ± 6 ng/dl, T\textsubscript{4} 4.2 ± 0.4 µg/dl, and CS: T\textsubscript{3} 47 ± 4 ng/dl, T\textsubscript{4} 3.9 ± 0.4 µg/dl). At the end of the 3-wk period, body weights of the CS animals were significantly lower than Ctl weights (\(P < 0.05\)), but they were comparable to the Sham animals (final body weights, 327.2 ± 8.8 g for CS, 337.5 ± 9.5 g for Sham, and 397.3 ± 3.4 g for Ctl).

Muscle fiber diameters. In all three experimental groups, there were significant differences in Di\textsubscript{mus} fiber diameters across fiber types (Table 1). In Ctl and Sham groups, the diameters of type I Di\textsubscript{mus} fibers were the smallest, followed in rank order by types I\textsuperscript{a} and II\textsuperscript{bx} (\(P < 0.05\); Table 1). In the CS-treated animals, the diameters of type I and I\textsuperscript{a} fibers were not significantly different, although type II\textsuperscript{bx} fibers remained larger (\(P < 0.05\); Table 1). The diameters of type II\textsuperscript{bx} fibers in the CS Di\textsubscript{mus} were significantly smaller than those of type II\textsuperscript{bx} fibers in both Ctl and Sham animals (\(P < 0.05\); Table 1). The diameters of type II\textsuperscript{bx} fibers in the Sham group were also smaller than those of Ctl animals (\(P < 0.05\); Table 1).

Nerve terminal morphology. In each experimental group, the planar (2-D) areas of nerve terminals innervating type I and IIa fibers were comparable but smaller than that of nerve terminals innervating type II\textsuperscript{bx} fibers (\(P < 0.05\); Table 1 and Fig. 2). The planar areas of nerve terminals innervating type I and IIa fibers were not significantly different across experimental groups, while the planar area of nerve terminals innervating type II\textsuperscript{bx} fibers varied significantly. In Sham animals, the planar area of nerve terminals innervating type II\textsuperscript{bx} fibers was significantly larger than that of Ctl (\(P < 0.05\)), whereas, in CS animals, type II\textsuperscript{bx} fiber nerve terminal area was significantly smaller than in both Ctl and Sham animals (\(P < 0.05\); Table 2).

When normalized for fiber diameter, the planar areas of nerve terminals innervating different fiber types in Ctl animals displayed a rank order, with type I > II\textsuperscript{a} > II\textsuperscript{bx} (\(P < 0.05\); Table 2). This rank order in normalized nerve terminal planar area was essentially reversed in CS and Sham animals. In CS, the planar area of nerve terminals innervating type II\textsuperscript{bx} fibers was significantly smaller than that of Ctl (\(P < 0.05\)), whereas, in Sham animals, type II\textsuperscript{bx} fiber nerve terminal area was significantly smaller than in both Ctl and Sham animals (\(P < 0.05\); Table 2).

Table 1. Effect of corticosteroid treatment on Di\textsubscript{mus} fiber diameter

<table>
<thead>
<tr>
<th>Group</th>
<th>Type I Diameter, µm</th>
<th>Type I\textsuperscript{a} Diameter, µm</th>
<th>Type II\textsuperscript{bx} Diameter, µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctl</td>
<td>26.2 ± 1.4</td>
<td>31.1 ± 1.7†</td>
<td>65.7 ± 2.7†</td>
</tr>
<tr>
<td>Sham</td>
<td>21.4 ± 1.1*</td>
<td>27.5 ± 1.3†</td>
<td>46.4 ± 3.8*</td>
</tr>
<tr>
<td>CS</td>
<td>24.7 ± 1.8</td>
<td>28.5 ± 2.2</td>
<td>34.2 ± 3.5†‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. Di\textsubscript{mus}, diaphragm muscle; Ctl, control; Sham, sham operated; CS, corticosteroid treated. *Significant difference from Ctl; †significant difference from type I; ‡significant difference from type II\textsuperscript{a}; §significant difference from Sham. Significance level is at \(P < 0.05\).
both Sham and CS animals, in which the normalized nerve terminal area of type IIx/b fibers was significantly greater than that of both type I and IIa fibers (P < 0.05; Table 2). Compared with Ctl, the normalized nerve terminal area of type IIx/b fibers was significantly larger in Sham and CS groups (P < 0.05; Table 2). However, in CS-treated animals, this increase in normalized nerve terminal area was due to the disproportionate reduction of fiber diameter, because nerve terminal area decreased (Tables 1 and 2). In contrast, in Sham animals, the increase in normalized nerve terminal area resulted from both a decrease in fiber area as well as an increase in nerve terminal planar area (Tables 1 and 2).

In Ctl and Sham animals, the total number of nerve terminal branches displayed a rank order across different fiber types, with type I < IIa < IIx/b (P < 0.05; Table 2 and Fig. 2). In Ctl animals, the total number of nerve terminal branches at type IIx/b fibers was also significantly greater than that at type I and IIa fibers (P < 0.05; Table 2), but there was no difference between type I and IIa fibers. In the CS group, the total number of nerve terminal branches on type IIx/b fibers was significantly lower, compared with Ctl (P < 0.05; Table 2).

In Ctl animals, the total cumulative length of all nerve terminal branches displayed a rank order across different fiber types, with type I < IIa < IIx/b (P < 0.05; Table 2). In Ctl and Sham animals, the total cumulative length of type IIx/b nerve terminal branches was significantly reduced compared with both Ctl and Sham groups (P < 0.05; Table 2). In the Ctl Di mus, the mean individual branch length of nerve terminals at type I fibers was greater than that of nerve terminals at type IIa and IIx/b fibers (P < 0.05; Table 2). In the CS group, mean individual nerve terminal branch lengths at type I and IIa fibers were comparable but remained significantly greater than that of nerve terminals at type IIx/b fibers (P < 0.05; Table 2). Within each fiber type, there was no significant difference between Ctl, Sham, and CS groups in the mean individual branch length of nerve terminals.

Motor endplate morphology. In all experimental groups, the planar areas of motor endplates at different fiber types displayed a rank order with type I < IIa < IIx/b (P < 0.05; Table 3 and Fig. 2). The planar areas of

### Table 2. Morphometric analysis of nerve terminals at different Di mus fiber types

<table>
<thead>
<tr>
<th>Group</th>
<th>Type I</th>
<th>Type IIa</th>
<th>Type IIx/b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Planar area, µm²</td>
<td>343 ± 19</td>
<td>370 ± 12</td>
<td>656 ± 12†‡</td>
</tr>
<tr>
<td>Normalized planar area, µm</td>
<td>13 ± 0.6</td>
<td>12.4 ± 0.8†</td>
<td>10.1 ± 0.9†‡</td>
</tr>
<tr>
<td>Total no. of branches</td>
<td>6 ± 1</td>
<td>10 ± 1†</td>
<td>24 ± 2†‡</td>
</tr>
<tr>
<td>Total branch length, µm</td>
<td>105 ± 7</td>
<td>123 ± 8†</td>
<td>224 ± 10†‡</td>
</tr>
<tr>
<td>Individual branch length, µm</td>
<td>17.5 ± 1.4</td>
<td>13.2 ± 1.1†</td>
<td>9.5 ± 1.0†‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Significant difference from Ctl; †significant difference from type I; ‡significant difference from type IIa; §significant difference from Sham. Significance level is at P < 0.05.

### Table 3. Morphometric analysis of motor endplates at different Di mus fiber types

<table>
<thead>
<tr>
<th>Group</th>
<th>Type I</th>
<th>Type IIa</th>
<th>Type IIx/b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Planar area, µm²</td>
<td>382 ± 18</td>
<td>420 ± 11†</td>
<td>830 ± 17†‡</td>
</tr>
<tr>
<td>Normalized planar area, µm</td>
<td>14.6 ± 0.8</td>
<td>14.0 ± 0.9</td>
<td>12.7 ± 1.4</td>
</tr>
<tr>
<td>Total no. of branches</td>
<td>6 ± 2</td>
<td>10 ± 1†</td>
<td>24 ± 3†‡</td>
</tr>
<tr>
<td>Total branch length, µm</td>
<td>109 ± 13</td>
<td>132 ± 9</td>
<td>230 ± 10†‡</td>
</tr>
<tr>
<td>Individual branch length, µm</td>
<td>18.3 ± 1.5</td>
<td>13.5 ± 1.2†</td>
<td>9.8 ± 1.4†‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Significant difference from Ctl; †significant difference from type I; ‡significant difference from type IIa; §significant difference from Sham. Significance level is at P < 0.05.
motor endplates at type I and IIa fibers were comparable among the three groups. However, the planar area of endplates at type IIx/b fibers of the CS Dimus was significantly smaller compared with that in both Ctl and Sham animals (P < 0.05; Table 3).

When normalized for fiber diameter, the planar areas of motor endplates at different fiber types were comparable in the Ctl Dimus (Table 3). In both Sham and CS groups, the normalized endplate areas at type IIx/b fibers were significantly greater than those at type I and IIa fibers (P < 0.05; Table 3), which were comparable to each other in both groups. In the CS Dimus, the normalized endplate area at type IIx/b fibers was significantly greater than that in Ctl (P < 0.05; Table 3). The normalized endplate areas at type I and IIa fibers were not different across experimental groups (Table 3).

In Ctl and Sham animals, the number of endplate branches displayed a rank order across different fiber types, with type I < IIa < IIx/b (P < 0.05; Table 3). In the CS group, the number of endplate branches was also greater at type IIx/b fibers compared with type I and IIa fibers (P < 0.05; Table 3). The number of endplate branches at type I and IIa fibers was comparable across the three experimental groups. However, the total number of endplate branches at type IIx/b fibers was significantly smaller in the CS group compared with Ctl (P < 0.05; Table 3).

In all experimental groups, the total cumulative length of endplate branches was comparable between type I and IIa fibers but was significantly greater at type IIx/b fibers (P < 0.05; Table 3). Total cumulative endplate branch length at type I and IIa fibers was comparable across the three experimental groups. However, the total cumulative branch length at type IIx/b fibers was significantly lower in the CS Dimus compared with Ctl and Sham animals (P < 0.05; Table 3). In the Ctl Dimus, the mean individual branch length of endplates displayed a rank order across different fiber types, with type I > IIa > IIb (P < 0.05; Table 3). In Sham animals, the mean individual branch length of endplates at type I fibers were longer than those at type IIa and IIx/b fibers (P < 0.05; Table 3). In the CS Dimus, there were differences in mean individual endplate branch lengths across the different fiber types (Table 3). Across the three experimental groups, there were no significant differences in individual branch lengths at any fiber type (Table 3).

Extent of overlap between nerve terminals and motor endplates. In Ctl animals, the extent of overlap between nerve terminals and motor endplates varied significantly across fiber types, with the overlap being ~95% at type I fibers, ~90% at type IIa fibers and ~80% at type IIx/b fibers (P < 0.05; Fig. 3). In CS animals, the extent of overlap between nerve terminals and motor endplates was unchanged at type I and IIa fibers, but the overlap was significantly greater at type IIx/b fibers, compared with Ctl (P < 0.05; Fig. 3).

Neuromuscular transmission failure. During the 2-min period of repetitive stimulation, the D_{infus} forces induced by both nerve and direct muscle stimulation decreased (Figs. 4 and 5). In the CS-treated animals, the difference between forces generated by nerve vs. direct muscle stimulation was less than that observed in both Ctl and Sham animals (Fig. 5). After 2 min, the decrement in force during nerve stimulation was comparable across all groups (Table 4). However, the decrement in force generated by direct muscle stimulation after 2 min was significantly greater in the CS-treated animals compared with both Ctl and Sham groups (P < 0.05; Fig. 5 and Table 4). Based on these differences in force generated by nerve vs. direct muscle stimulation, it was estimated that the relative extent of neuromuscular transmission failure was significantly smaller in the CS Dimus compared with the extent in both Ctl and Sham animals (P < 0.05; Table 4).

DISCUSSION

The major observations of the present study were that 3 wk of prednisolone treatment resulted in remodeling of pre- and postsynaptic elements of NMJ's at type IIx/b Dimus fibers and an improvement in neuromuscular transmission. At type IIx/b fibers, nerve terminal and endplate areas decreased with CS treatment, whereas NMJ's on type I and IIa fibers were largely unaffected. The reduction in NMJ area at type IIx/b fibers in the CS-treated Dimus resulted from a decrease in the number of nerve terminal and endplate branches and a shorter mean branch length. However, the decrease in NMJ size at type IIx/b fibers with CS treatment was proportionately less than the fiber atrophy. Thus, with CS treatment, NMJ size normalized for fiber diameter actually increased at type IIx/b fibers compared with Ctl. Furthermore, the extent of overlap between nerve terminal and motor endplate increased at type IIx/b fibers of the CS Dimus. Because type IIx/b fibers are normally more susceptible to neuromuscular
transmission failure (17), these selective changes in NMJ morphology at type IIx/b fibers were consistent with the improvement in neuromuscular transmission observed in the CS-treated Dimus.

CS-treatment regimen. The daily prednisolone dose used in the present study was comparable to that used in other studies (4, 42–44). However, as in our previous study (42), prednisolone was continuously infused via a miniosmotic pump, which undoubtedly maintains a more stable serum level of CS compared with bolus injections. A comparison of the serum prednisolone levels obtained in the present study in rats with therapeutic levels in humans is difficult because of the likelihood of different pharmacokinetics and pharmacodynamics between rats and humans, and because serum prednisolone levels are infrequently reported. Furthermore, comparison of daily doses is complicated by the varying modes of administration in the clinical environment. However, a recent pharmacokinetic study reported that, after multiple oral administrations in human subjects, stable serum prednisolone levels were achieved that were comparable to those in the present study (36).

The selection of the 3-wk experimental period in the present study was entirely arbitrary and was largely based on the time period selected in our previous study (42). Certainly, both acute and chronic CS treatment are used clinically. The efficacy of prednisolone treatment in rats was evident by a reduction in body weight of ~20%, which was comparable to the weight loss observed in previous studies conducted over a similar 3-wk period (11, 24, 47).

Effects of CS on Dimus fiber morphology. The observation that CS treatment was associated with a selective atrophy of type IIx/b fibers in the rat Dimus was consistent with several previous reports both in the Dimus (24, 42, 45, 47) as well as in hindlimb muscles (14, 45). However, the results of the present study contrast with those reported in the rabbit Dimus, in which cortisone treatment for 3 mo was found to be associated with a greater atrophy of type I fibers (11). This apparent discrepancy cannot be fully explained, but it may reflect species differences in the response to CS treatment. Furthermore, there may be differences between the effects of prednisolone (a nonfluorinated CS) vs. cortisone (which is fluorinated). Yet another factor that needs to be considered is the time course for CS effects, which may differ across species and/or the type of CS.
agent used. In the present study, CS effects on the rat DI\textsubscript{mus} were assessed only after 3 wk.

The differential effect of CS treatment on type IIx/b fibers most likely reflects a selective inhibition of protein synthesis and/or an enhancement of protein degradation in these fibers (25). The underlying mechanisms for this selective catabolic effect remain unknown. One possibility is that fiber type differences exist in glucocorticoid receptor expression. However, in a previous study, it was reported that muscles that are predominantly composed of type I fibers have a higher concentration of glucocorticoid receptors, compared with muscles that are predominantly composed of type II fibers (19). However, fiber type differences in glucocorticoid receptor expression in the rat DI\textsubscript{mus} have not yet been characterized.

Effects of CS on NMJ morphology. In the Ctl DI\textsubscript{mus}, the planar areas of nerve terminals and motor endplates at type I and IIa fibers were significantly smaller than the planar area of NMJ s at type IIx/b fibers. This result is consistent with our previous observations in the rat DI\textsubscript{mus} (31, 32) and with other studies in the DI\textsubscript{mus} (46) as well as limb muscles (10, 41, 46). Also, consistent with our previous observations, we found that NMJ size normalized for muscle fiber diameter was actually greater at type I and IIa fibers compared with type IIx/b fibers. Moreover, as reported earlier (31, 32), we found that the branching patterns of NMJ s at type I and IIa fibers were relatively simple and compact compared with the more rosette and elaborate patterns of NMJ s at type IIx/b fibers.

Previous studies in various muscles have also demonstrated a correlation between muscle fiber diameter and NMJ size (27, 28, 32). In fact, it has been suggested that muscle fiber size is a major determinant of NMJ size and that changes in muscle fiber size actually trigger changes in NMJ morphology (27, 28, 32). Our previous study (46) in the rat DI\textsubscript{mus} demonstrated that the correlation between muscle fiber diameter and NMJ size is valid only within a fiber type, hence the differences in normalized NMJ size across fiber types. However, we observed that changes in NMJ size were not always associated with changes in muscle fiber diameter (35). For example, 2 wk of DI\textsubscript{mus} hemiparalysis induced by cervical spinal cord hemisection were associated with a significant expansion of NMJ size at type IIx/b fibers in the absence of any significant change in muscle fiber size (35). Conversely, 2 wk of DI\textsubscript{mus} hemiparalysis induced by tetrodotoxin blockade of phrenic nerve axonal conduction resulted in substantial atrophy of type IIx/b fibers and hypertrophy of type I and IIa fibers, without concomitant changes in NMJ morphology (33). In the present study, the selective atrophy of type IIx/b fibers induced by CS treatment was associated with a reduction in NMJ size at these fibers. However, the reduction in NMJ size did not match the atrophy of type IIx/b fibers in the CS-treated DI\textsubscript{mus}. Indeed, type IIx/b NMJ size normalized for muscle fiber diameter was actually greater compared with Ctl.

In previous studies by Fahim and colleague (7, 8), fiber type differences in the effects of CS treatment on NMJ morphology were evaluated by comparing NMJ s at fibers in the soleus (type I fibers) vs. extensor digitorum longus (type II fibers) muscles. In one study (8), it was reported that 3 mo of cortisone treatment resulted in an enlargement of nerve terminals at type I fibers in the soleus muscle, with little or no structural changes in nerve terminals at type II fibers in the extensor digitorum longus muscle. However, in a subsequent study (7), Fahim reported that 3 mo of cortisone treatment resulted in a reduction in nerve terminal size, and the reduction was more pronounced in type II fibers in the extensor digitorum longus muscle. It was suggested that the apparent discrepancy between the results of these two studies could be attributed to the dynamic nature of NMJ remodeling, with simultaneous degeneration and regeneration of nerve terminals taking place (7), akin to that seen with aging (9).

It is likely that, in the CS-treated DI\textsubscript{mus}, the macroscopic changes in NMJ morphology at type IIx/b fibers were also accompanied by a reduction in the number of nerve terminal active zones and/or the number of synaptic vesicles. However, these ultrastructural changes could not be resolved at the light-microscopic level. Previous ultrastructural studies have reported that CS treatment is associated with changes in synaptic vesicle size, vesicular density, and synaptic cleft width (7, 22, 23, 44). Glucocorticoids have also been reported to increase acetylcholinesterase levels at NMJ s on innervated, cultured human skeletal muscle (3). Furthermore, electrophysiological studies have reported changes in MEPP amplitude after CS administration (48). Thus it appears that CS treatment induces both gross and ultrastructural changes at the NMJ that may affect neuromuscular transmission. The extent of neuromuscular transmission failure was estimated by comparing the decrement in force during repetitive nerve stimulation to the force induced by direct muscle stimulation. There are several assumptions made in using this method. For example, it must be assumed that differences in stimulation parameters between nerve and direct muscle stimulation (e.g., pulse duration, current intensity) do not artifically affect the forces generated. For example, the longer pulse duration and higher current intensity used during direct muscle stimulation may lead to multiple force responses during a single pulse. However, it is extremely doubtful that such multiple force responses would occur if a pulse duration of 1 ms were used. At a pulse duration of 0.2 ms, as used for nerve stimulation, we found that stimulus current intensities exceeding 750 mA are required for supramaximal direct muscle stimulation, compared with a current intensity of ~225 mA at a pulse duration of 1 ms. A higher current intensity could cause serious muscle fiber injury, especially during repeated stimulation.

A second factor that complicates interpretation of the present results is the fact that CS treatment may affect fatigue resistance of the DI\textsubscript{mus} to repetitive direct stimulation.
muscle stimulation. However, previous studies have been equivocal in this regard, with some studies reporting an improvement in fatigue resistance (26, 42, 47), whereas other studies have reported greater fatigability (12, 24). Using the same mode of continuous administration of prednisolone, we previously observed that CS treatment had only a relatively modest effect on \( D_{\text{mus}} \) fatigue during repetitive isometric shortening induced by direct muscle stimulation (42). Furthermore, direct muscle stimulation was imposed every 15 s, compared with more frequent activation in previous studies that assessed fatigability (e.g., stimulus trains repeated each second). Therefore, it is doubtful that differences in fatigue induced by direct muscle stimulation influenced the estimates of neuromuscular transmission failure.

The results of the present study indicated that the extent of neuromuscular transmission failure was lower in the CS-treated \( D_{\text{mus}} \) compared with Ctl. Neuromuscular transmission failure during repetitive nerve stimulation has been attributed either to a failure in the axonal propagation of action potentials, primarily at axonal branch points, or to a failure of synaptic transmission, at either pre- or postsynaptic sites (13, 20, 38, 40). The improvement in neuromuscular transmission after CS treatment could be attributed to either mechanism. In the normal \( D_{\text{mus}} \), type IIx/b fibers are more susceptible to neuromuscular transmission failure, compared with type I or IIa fibers (17). This is consistent with the morphology of NMJ s at type IIx/b fibers which have a greater number of nerve terminal branches and less overlap between pre- and postsynaptic elements, compared with NMJ s at type I and IIa fibers (32). After CS treatment, the total number of nerve terminal branches was reduced at type IIx/b fibers. Thus the probability of axonal branch point failure may have decreased at type IIx/b fibers. In addition, the extent of overlap between nerve terminals and motor endplates improved at type IIx/b fibers after CS treatment. In areas of the NMJ where the motor endplate extends beyond nerve terminals (i.e., regions of nonoverlap), the ACh released at nerve terminals may be less effective in inducing postsynaptic membrane potential changes. Accordingly, the extent of overlap between pre- and postsynaptic elements after CS treatment may contribute to an improvement in synaptic efficacy.

The disproportionate effect of CS treatment on type IIx/b fiber diameter vs. NMJ size may also contribute, at least in part, to an improvement in neuromuscular transmission. Larger muscle fibers have greater total capacitance, requiring greater synaptic current (quantal ACh release) to generate an action potential (lower safety factor). Thus type I and IIa fibers, with smaller fiber cross-sectional areas and greater normalized size of NMJ s, have higher safety factors for neuromuscular transmission than do type IIx/b fibers. With CS-related atrophy of type IIx/b fibers, the safety factor for synaptic transmission would improve by offsetting the point at which quantal release of ACh is insufficient to generate an action potential.

In conclusion, the present study found that CS administration leads to a selective atrophy of type IIx/b fibers and a disproportionate reduction in NMJ size at these fibers. The morphological alterations of NMJ s at type IIx/b fibers would lead to lower probability of axonal branch point failure and greater synaptic efficacy. Accordingly, it was observed that CS treatment was associated with decreased neuromuscular transmission failure in the \( D_{\text{mus}} \).

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