Resistance to d-Tubocurarine in Lower Motor Neuron Injury Is Related to Increased Acetylcholine Receptors at the Neuromuscular Junction

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The hypothesis that lower motor neuron injury, with its associated proliferation of acetylcholine receptors (ACHR), induces resistance to the neuromuscular effects of d-tubocurarine (dTC) was tested in the rat. The left gastrocnemius was denervated by a 75–80% lesion of the sciatic nerve. The effective dose for 95% twitch depression (ED95) was studied in the denervated gastrocnemius and compared to the contralateral undenervated and sham-injured (control) gastrocnemius muscles approximately 2 weeks after injury. The AChR number was quantitated by the specific ligand 125I-α-bungarotoxin (125I-α-BT). Plasma dTC concentrations, measured by high-performance liquid chromatography (HPLC), were correlated to twitch tension during spontaneous recovery from neuromuscular blockade in the denervated animal. The ED95 (mean ± SE) of dTC for the denervated leg was significantly (P < 0.05) higher (0.26 ± 0.06 mg·kg⁻¹) than contralateral (0.16 ± 0.03) and sham-operated left (0.13 ± 0.03) legs. The twitch tension recovered to 50% of control twitch height at significantly (P < 0.05) higher plasma dTC concentrations in the denervated (0.78 µg·ml⁻¹) compared to contralateral (0.24 µg·ml⁻¹) limb. The AChR number was significantly increased in the denervated limb (1041 ± 96 fmol·mg protein⁻¹) compared to contralateral right (105 ± 4) and control left limb (113 ± 11). There was a significant (P < 0.05) positive correlation (R² = 0.73) between ED95 and AChR number; that is, 73% of the variability in ED95 could be explained by changes in AChR. This study, therefore, confirms the hypothesis that proliferation of AChR after nerve denervation results in resistance to the neuromuscular effects of dTC. Since a partial lower motor neuron lesion of one side does not affect contralateral neuromuscular responses, the contralateral normal side can be used to assess neuromuscular function, and therefore contrasts with reports on upper motor neuron denervation in which altered sensitivity to neuromuscular relaxants may occur on the affected as well as “unaffected” skeletal muscles. (Key words: Complications: denervation; lower motor neuron injury. Injury: lower motor neuron.

Neuromuscular relaxants: d-tubocurarine. Receptors: acetylcholine receptors; nicotinic receptors.)

ABNORMAL RESPONSES TO neuromuscular blocking drugs after burn trauma and upper motor neuron injury have been described.1-6 Common to both conditions is a proliferation of acetylcholine receptors (ACHR) on the skeletal muscle.7-10 Pharmacologic investigations of drug-receptor interactions in other receptor models (e.g., adrenergic receptors) reveal that there is hypersensitivity to receptor agonists (e.g., epinephrine) and hyposensitivity to competitive antagonists (e.g., propranolol) when receptor numbers are increased.11 (Throughout this article, "hypersensitivity" and "hyposensitivity" refer to a shift to the left and right, respectively, of the dose or concentration–response curves.) With respect to the nicotinic AChR, the hypersensitivity to agonists has been confirmed, where one tenth of normal dose of acetylcholine depolarizes skeletal muscle in the presence of increased AChR numbers.8 Supersensitivity to the AChR agonist succinylcholine also occurs in burns and denervation states where the concentrations of AChR are increased.5,12,13 Increased receptor spread is believed to be responsible for the exaggerated potassium release when succinylcholine is given to patients with burns or denervation injuries.5 Complementing these responses to agonist drugs is the observation that resistance (hyposensitivity) to the effects of antagonists or competitive drugs (e.g., d-tubocurarine [dTC]) occurs in burns and upper motor neuron lesions. The relationship between the increased dose requirement for dTC to elicit twitch depression and AChR changes has been confirmed in the rat model of burn injury.10

Lower motor neuron injury induces AChR proliferation and super sensitivity responses to agonist drugs such as acetylcholine and succinylcholine.12-14 Using the rat model, the current study tested the hypothesis that lower motor neuron injury-induced increases in AChR result in resistance to competitive antagonist neuromuscular blockers. The most extensively studied and used pharmacologic probe of the neuromuscular junction, dTC, was used as the test drug. Neuromuscular responses to dTC and changes in AChR were studied in the denervated and contralateral undenervated gastrocnemius muscles and were compared to sham-operated controls.
Materials and Methods

Nerve Injury

Experiments performed in this study adhered to National Institutes of Health and institutional animal care guidelines. Eighteen male Sprague-Dawley rats (Charles River, Boston, MA) weighing 200–250 g were anesthetized with 50 mg·kg⁻¹ pentobarbital administered intraperitoneally, with subsequent incremental doses administered as needed. The left hind limb was shaved and prepared with an iodine solution. A longitudinal incision was made along the posterior aspect of the limb, and the sciatic nerve was exposed by dissection. In 9 animals an approximate 75–80% transection of the sciatic nerve was performed, ensuring that some continuity of the nerve was maintained. Transection of the whole nerve, which results in Wallerian degeneration, was avoided since it abolishes nerve conduction and evoked twitch responses, both of which are necessary for the subsequent neuromuscular pharmacodynamic studies. In the remaining 9 animals, the sciatic nerve was exposed but the nerve was not transected. All incisions were sutured with 2-0 silk, and an antibiotic ointment was applied to the incision. Animals were returned to their cages, and all received identical postoperative care.

Dose–Response Determination

The neurovascular responses to dTC were studied in the control and experimental groups at a mean (± SE) of 12.0 ± 0.53 and 15.6 ± 0.5 days, respectively, after the operation. On the day of the experiment each rat was anesthetized with pentobarbital as described above. A tracheostomy was performed, and venous access was obtained by cannulation of the jugular vein. The animal’s lungs were ventilated with room air using a Harvard ventilator with ventilation adjusted to maintain physiologic venous blood gases. Rectal temperature was monitored and maintained between 35–37°C with a heat lamp.

A longitudinal incision was made along the posterior aspect of the thigh and leg to isolate the gastrocnemius muscles and sciatic nerves on both sides of each animal. Each tendon of the gastrocnemius muscle was attached to a Grass FT03 force transducer with 2-0 silk. The baseline tension was set at 50 g for all right gastrocnemius muscles and 10 g for all left gastrocnemius muscles. Stimulating electrodes were placed on the sciatic nerves, and in the case of the partially transected sciatic nerve, the electrode was placed distal to the site of previous transection. Electrical stimulation of the sciatic nerve was carried out with supramaximal pulses of 0.15 Hz and 0.2-ms duration. Twitch responses of the gastrocnemius muscles on both legs induced by electrical stimulation of the sciatic nerve were recorded on a Western Graphtec WR 7500 recorder. The twitch tension responses were allowed to stabilize to the stimulation for at least 10 min, after which incremental doses of dTC were administered intravenously to achieve 95–99% twitch suppression of the right gastrocnemius muscle.

Plasma dTC Concentration

After 95–99% twitch suppression was achieved, the muscles were allowed to recover spontaneously. At various points during recovery from paralysis, venous blood samples were obtained from the denervated animals via the jugular vein to measure plasma dTC concentrations. The twitch tension at the time of blood sampling was noted. The plasma samples were stored at −80°C and later analyzed for dTC concentrations with high-performance liquid chromatography (HPLC).¹⁵

AChR Assay

Upon recovery of twitch suppression the animals were killed with an overdose of pentobarbital. Pieces of the right and left gastrocnemius muscles (1 × 1 cm) were excised at the point of entry of the sciatic nerve. Similar-size pieces of right and left hemidiaphragms with their respective phrenic nerves attached also were obtained. Each muscle was immediately washed three times in a 50 mM phosphate buffer (pH 7.4) containing 0.1 mM benzethonium chloride and 1 mM EDTA, rinsed in saline, and frozen immediately at −80°C in separate glass jars.

On the day of the AChR assay the gastrocnemius and diaphragm muscles were thawed and washed three times in the same 50 mM phosphate buffer. The nerves were discarded, each skeletal muscle was homogenized, and AChR extraction from muscle membrane was performed by adding 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 1 mM EDTA, and 1.5% Triton X-100 detergent in 50 mM phosphate buffer at pH 7.4 (Sigma Chemical, St. Louis, MO).¹⁰,¹⁷ The mixture was shaken continuously for 14 h at 4°C. This procedure resulted in the extraction of 92.3 ± 1.1% of total AChR from the tissue.¹⁵ The samples were centrifuged at 5000 rpm for 40 min at 4°C, and the supernatants were removed.

¹²⁵I-α-bungarotoxin (¹²⁵I-α-BT), a specific ligand for nicotinic AChR, was used to quantify AChR at the neuromuscular junction.¹⁶,¹⁹ ¹²⁵I-α-BT (0.1 ml) (specific activity 16.2 μCi/μg; NEN Products, Boston, MA) was added to 0.5 ml of the supernatant after dilution of ¹²⁵I-α-BT to 0.5 μg/ml in 50 mM phosphate buffer and 1.5% Triton-X 100. The mixture was incubated at room temperature, shaken for 1 h, and frozen until column chromatography.¹⁶,¹⁷ In preliminary experiments, the efficacy...
of $^{125}$I-α-BT binding to AChR was tested with incubation times of 0.5, 1.0 and 2.0 h; total recovery of $^{125}$I-α-BT was the same at all three times. Thus, the concentration used (0.5 ml of 0.5 μg/ml $^{125}$I-α-BT solution) with an incubation time of 1 h was sufficient time for $^{125}$I-α-BT to form a complete complex with AChR. These results are consistent with other reports.\textsuperscript{16}

Specific and nonspecific binding of $^{125}$I-α-BT to muscle was measured as follows. The thawed muscle tissue was separated into two aliquots, both of which were incubated with $^{125}$I-α-BT. To one aliquot excess cold α-BT (Sigma Chemical) was added, and the mixture was incubated for an additional 1 h at room temperature. Specific binding of α-BT was calculated as the difference between bound radioactivity in the presence and absence of excess unlabeled ligand.

Column chromatography was used to separate $^{125}$I-α-BT bound to nicotinic AChR from free $^{125}$I-α-BT.\textsuperscript{16,17} Columns (1 × 25 cm) were packed with Sephadex G-50 (Pharmacia, Inc., Piscataway, NJ) and equilibrated with eluting buffer consisting of 0.1 M NaCl, 1 mM EDTA, 0.1 mM benzethonium chloride, and 1% Triton X-100 in 20 mM phosphate buffer at pH 7.4.\textsuperscript{17,19} Two tenths of 1 ml of each sample was added to the column. A Gilson fraction collector (FC-80K Fractionator; Gilson Medical Electronics, Middletown, WI) was used to collect 25 drops per tube, and the radioactivity was counted with the $^{125}$I channel of a gamma counter (Tracer Analytic; Elk Grove Village, IL). The first peak of the chromatogram represented $^{125}$I-α-BT-bound AChR. The efficiency of $^{125}$I counting was 75%, according to manufacturer specifications. The protein concentration of the muscle extract was determined with the Hartree assay method.\textsuperscript{20} The molar quantity of nicotinic AChR was calculated based on $^{125}$I-α-BT counts per minute using disintegrations per minute, specific activity, and molecular weights of $^{125}$I-α-BT. The number of AChR was expressed as fmol·mg protein$^{-1}$ of AChR in 1 ml of the extract.

**Statistical Analysis**

Dose–response curves to dTC were constructed on log–probit coordinates. The effective doses to produce 50 and 95% twitch suppression (ED$_{50}$ and ED$_{95}$, respectively) were calculated by the least-squares regression method. Significance between groups was determined by one-way analysis of variance, with $P < 0.05$ considered significant. The least-squares regression technique was used to correlate per cent twitch recovery to plasma dTC concentration. Examination of the data revealed no evidence of systematic departure from straight-line dependence of twitch suppression on plasma dTC levels. Thus, point estimates and confidence limits for plasma dTC concentrations that would give 50% twitch depression (Cp$_{50}$) were calculated by a straightforward modification of the linear calibration approach.\textsuperscript{21} The Cp values were declared significantly different at $P < 0.05$ if the intervals given by their respective 97.5% confidence limits did not overlap.

**Results**

**Dose–Response to dTC**

The time after operation at which neuromuscular pharmacodynamics was performed did not differ between experimental and control groups. The left gastrocnemius muscle of the animal whose sciatic nerve was subjected to the partial transection was grossly atrophied compared to both the contralateral right gastrocnemius of the same animal and the gastrocnemius muscles of controls. Prior to experiments in which the sciatic nerve was stimulated an attempt was made to set the baseline preload tension of the partially denervated muscle to 50 g; however, the atrophic muscle would not contract at this preload to allow recording of twitch response. Therefore, the baseline tensions of both the partially denervated left gastrocnemius and the left gastrocnemius muscle of the controls were set at 10 g. The right gastrocnemius muscle in the denervated and control animals was able to develop twitch tension at a preload of 50 g.

Figure 1 is a representative tracing of the simultaneous recording of the right and left gastrocnemius muscles in an animal in which the left sciatic nerve was partially transected. The onset of paralysis in the partially denervated left leg was slower than that of the uninjured right leg. The denervated left leg continued to have a twitch re-

![Figure 1](https://example.com/image.png)
TABLE 1. Effective Dose (ED) for dTC

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Injured Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right Leg</td>
<td>Left Leg</td>
</tr>
<tr>
<td>ED₉⁰ (mg·kg⁻¹)</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>ED₅₀ (mg·kg⁻¹)</td>
<td>0.15 ± 0.05</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>Preload (g)</td>
<td>50</td>
<td>10</td>
</tr>
</tbody>
</table>

Values given are mean ± SE.

* P < 0.05 denervated left versus control left or contralateral right leg.

The ED values for each leg in the two groups are shown in table 1. The denervated gastrocnemius showed resistance to the neuromuscular effects of dTC. An increased ED₉⁰ was seen not only when compared to the contralateral leg but also when compared to the left leg of control animals. The ED₅₀ and ED₉⁰ of right and left legs in controls were the same despite their different preloads, as were the ED₅₀ and ED₉₀ of right legs compared between groups (same preload).

PLASMA dTC CONCENTRATION–RESPONSE CURVES

The relationship between plasma dTC concentrations and twitch recovery for the denervated left leg compared to the contralateral right leg is shown in figure 2. The slopes and intercepts of the concentration–response curves were significantly different between the denervated left and contralateral right leg. The Cp₅₀ (with its lower and upper 95% confidence limits) of 0.78 (0.66–1.14) μg·ml⁻¹ for the denervated limb was significantly higher than the Cp₅₀ of 0.24 (−0.58 to 0.42) μg·ml⁻¹ for the contralateral limb. In other words, a greater degree of neuromuscular recovery occurred in the partially denervated left leg for a given plasma dTC concentration compared to the contralateral leg.

ACHR CHANGES

The ACHR changes in the right and left hemidiaphragms and gastrocnemius muscles for the experimental and control groups are shown in table 2. The denervated left gastrocnemius muscles had significantly higher concentrations of ACHR compared to control and contralateral gastrocnemius muscles. Comparison of the ACHR changes in the right contralateral leg of the denervated animal or left and right legs of controls did not show any differences. Therefore, the changes in ACHR in the gastrocnemius (table 2) paralleled the ED₉₀ responses of dTC to each of these muscles (table 1). The relationship between changes in ACHR and ED for control and partially denervated left legs, shown in figure 3, demonstrate a significant positive correlation; as the ACHR number increases, the ED₅₀ (R² = 0.47, r = 0.69) and ED₉₀ (R² = 0.73, r = 0.85) increase.

ACHR number increased in the left hemidiaphragms of the animals subjected to the lower motor neuron injury of the sciatic nerve, compared to ACHR number in the right hemidiaphragms of the same animals (table 2). The number of ACHR in the left hemidiaphragms of animals that underwent partial denervation of the sciatic nerve was also significantly increased when compared to right or left hemidiaphragms of the control group. No significant differences in ACHR number were observed between the right hemidiaphragms of the partially denervated group and those of the control group. The ACHR number in the right and left hemidiaphragms of the control group did not differ.

Discussion

The current study confirmed the hypothesis that an increase in ACHR induced by lower motor neuron denervation results in a resistance to the neuromuscular effects of dTC. Neuromuscular responses were tested on
DENERVATION INDUCED RESISTANCE TO d-TUBOCURARINE

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Control</th>
<th>Denervated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>104.4 ± 11.17</td>
<td>115.0 ± 11.17</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>47.0 ± 2.23</td>
<td>47.7 ± 2.73</td>
</tr>
</tbody>
</table>

Values given are mean ± SE.
* P < 0.05, left denervated limb compared to contralateral or control limbs.
** P < 0.05, left hemidiaphragm of denervated animals compared to contralateral hemidiaphragm or control right and left hemidiaphragms.

Intuitively, it is difficult to understand how a denervated and atrophied muscle will show resistance (hypersensitivity) to the effects of a drug that further weakens contractile properties. These observations, however, can be explained on a pharmacologic basis. The denervation process resulted in an increase in AChR (table 2). Because of the increase in AChR, more dTC was required for AChR occupancy and prevention of neuromuscular transmission. In other words, for a given plasma concentration, the percent occupancy of the AChR by dTC was decreased because of the increase in AChR number. This theory was confirmed by the significant positive correlation between AChR and ED₉₀ or ED₉₅ (R² = 47 and 73%, respectively) (fig. 3); it is 47 and 73% of the variability in ED₉₀ and ED₉₅ were explained by changes in AChR. Additional factors that may contribute to the resistance induced by injury include altered protein binding of dTC, changes in acetylcholinesterase enzyme activity, changes in AChR receptor affinity, or all of these. The current data, documenting that increased AChR is associated with resistance to dTC, is consistent with those of another pathologic state, burns, for which increased ED values correlated well to increased AChR. The resistance to the competitive antagonist dTC documented in the current study and the documented hypersensitivity to the agonist succinylcholine after denervation, in which AChR concentrations are increased, contrasts with neuromuscular responses observed in another pathologic state, myasthenia gravis, where AChR number is decreased. In myasthenia gravis, the decrease in AChR results in hypersensitivity to dTC and resistance to succinylcholine.

After the administration of a dose of drug there is a temporal disequilibrium between plasma concentration and pharmacodynamic effect. In contrast, if a continuous infusion is maintained, this disequilibrium is obviated after both a steady-state plasma concentration and distribution equilibrium are obtained. In these instances, the plasma concentrations of the drug should reflect the concentrations at the site of action, and a different plasma concentration-response relationship between two end organs (sites) is likely to represent an actual difference in pharmacodynamics. If steady-state conditions are not present, the pseudo-equilibrium that occurs during ter-
minal elimination phase is an approximation of steady state.\textsuperscript{27,28} In our study, we assumed that during recovery of twitch tension there is a pseudo-equilibrium between the concentration of dTC in plasma and that at the AChR site. Hence, the significantly higher C\textsubscript{p} value for the denervated side compared to contralateral side indicates altered pharmacodynamics.\textsuperscript{28}

Normally only the junctional receptors are involved in neuromuscular transmission since the number of extra-junctional receptors is insignificant. The pathologic process of denervation incorporates new and increased receptor sites both at junctional and extra-junctional areas,\textsuperscript{29} but the role of junctional versus extra-junctional receptors in neuromuscular transmission in these pathologic states is unclear.\textsuperscript{2,8} In our assay for AChR we did not attempt to distinguish between junctional and extra-junctional receptors. The muscle sample assayed (1 \times 1-cm piece) was taken from the point of entry of the nerve in both the diaphragm and gastrocnemius. Therefore, the muscle tissue assayed is probably more representative of junctional rather than extra-junctional receptors.

It is known that upper motor neuron lesions also induce a proliferation of AChR.\textsuperscript{7,30} The neuromuscular pharmacodynamic studies in patients with upper motor lesions (e.g., stroke or spinal cord injury) document an increased dose or plasma concentration requirement of nondepolarizing relaxant compared to the requirements of controls.\textsuperscript{1,5,7} In all of these studies, systematic evaluation of the role AChR plays in altered dose or plasma concentration requirement was not undertaken. The findings in the current study of lower motor neuron injury on the pharmacodynamics of dTC differ in a number of ways from the responses observed in upper motor neuron lesion. Upper motor neuron lesions generally have bilateral or generalized alterations in neuromuscular response. In patients who have had a stroke, Shayevitz and Matteo, for example, showed an increased metocurine plasma concentration requirement for neuromuscular paralysis compared to the requirement of controls.\textsuperscript{2} This increased metocurine requirement was seen not only on the affected side, but also on the opposite side. This result contrasts with our study in that the contralateral undenervated side of the denervated animal demonstrated no change in ED or AChR. Brett \textit{et al.},\textsuperscript{7} in a study of one patient with upper motor neuron lesion (multiple sclerosis), documented the need for higher infusion rates of atracurium to maintain twitch paralysis compared to controls; that is, the disease process affected total infusion rates. In our study, the ED and plasma concentrations were increased only for the affected (denervated) leg, whereas dTC requirements for the contralateral side were unaltered. In other words, the increase in AChR on the denervated muscle was not acting as a sink for accumulating dTC and thereby was not increasing ED of normally innervated muscle. Even if the denervated muscle was functioning as a reservoir for dTC (by increased specific and nonspecific binding), this may increase ED or total drug administered but not necessarily C\textsubscript{p} for the normal side.

Steinbach reported in a cat model that motor denervation of one extremity resulted in changes in AChR on the affected side as well as on the opposite, normally innervated leg.\textsuperscript{14} These results, along with the findings of bilateral involvement of resistance to nondepolarizing muscle relaxants in upper motor neuron lesions,\textsuperscript{2,5,7} are the reason we used a separate group of control animals and not the contralateral leg of the experimental group as the control. We reasoned that if AChR numbers vary in the opposite, normally innervated extremity of the animals whose left sciatic nerves were partially denervated, then the ED of dTC may also be affected. Hence, the contralateral leg would not serve as a true control. In contrast to Steinbach’s findings, however, AChR concentrations in the contralateral leg of our model were not significantly different from controls. Numerous factors may have resulted in the disagreement between the two studies. Steinbach used a cat rather than rat model, and the motor nerve was completely transected compared to partially transected, as in our study. In addition, the complete inactivity of the denervated muscle of the cats may have caused disuse of the opposite leg. Disuse atrophy increases AChR\textsuperscript{51} and results in resistance to nondepolarizing muscle relaxants.\textsuperscript{52} AChR from the diaphragms of both control and experimental groups were studied to evaluate any systemic proliferation of AChR with a localized injury, as was seen in burn trauma.\textsuperscript{9,10} The use of the diaphragm to study AChR eliminates the question of disuse atrophy as a variable in AChR changes. A novel and unexpected finding in the current study was the increase in AChR in the left but not the right hemidiaphragm of the denervated animals. This finding should be confirmed by future experiments; although the exact reason is unclear, it is tempting to speculate that this increase in AChR in the left hemidiaphragm may be the result of retrograde demyelination affecting the same side of the spinal cord.

The implications of the current study are particularly relevant to the clinical anesthesiologist. Not only is lower motor neuron injury common, but also patients with these injuries require repeated anesthetics for related trauma. Because of the concern for avoiding hyperkalemia after depolarizing relaxants, nondepolarizing relaxants are the muscle relaxants of choice. From our study we can extrapolate that uninvolved muscle will have a normal response to nondepolarizing muscle relaxants but that denervated muscle will be resistant. During clinical anesthesia, monitoring of neuromuscular blockade with a peripheral nerve stimulator should be performed on extremities that are not involved in neuronal injury. Mon-
itoring neuromuscular blockade in an extremity that has a lower motor neuron injury will result in overdosing because of the resistance to the neuromuscular effects of a nondepolarizing relaxant on that affected side. Likewise, during the offset of paralysis, the resistant extremity may show adequate recovery, while normal muscle, including respiratory muscles, may not be fully recovered.

References