Prolonged d-Tubocurarine Infusion and/or Immobilization Cause Upregulation of Acetylcholine Receptors and Hyperkalemia to Succinylcholine in Rats

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Background: Hyperkalemic cardiac arrest after the administration of succinylcholine (SCH) to critically ill intensive care patients has been attributed to changes in the acetylcholine receptors (AChRs) at the muscle membrane. The current study attempts to characterize the contributory roles of chronic administration of nondepolarizing muscle relaxants typified by d-tubocurarine (dTC) and/or immobilization on AChR upregulation and the relationship of these AChR changes to SCH-induced hyperkalemia.

Methods: Rats received chronic subparalytic infusion of saline or dTC for 28 days via subcutaneous osmotic pumps inserted while they were under anesthesia. Approximately half of the saline- or dTC-treated rats underwent bilateral hindlimb immobilization with plaster casts for the same duration as the infusion. After 4 weeks, the osmotic pumps were removed, and 24–48 h later, the blood potassium concentrations were measured at baseline and at 1, 3, 5, 7, and 10 min after SCH (3 mg/kg). At the end of this period, the gastrocnemius muscle was excised for quantification of AChR number using 125I-O-bungarotoxin.

Results: At 28 days, the weight gain in mobile animals receiving saline or dTC infusion did not differ, nor did it in immobilized animals receiving saline or dTC infusion, confirming that infusion of dTC did not unduly affect the ability of the animals to feed. The maximal potassium change after SCH occurred at 5 min. Potassium responses to SCH changed (mean ± SE): (1) from 3.9 ± 0.4 to 4.5 ± 0.1 mEq/l in the mobile saline-treated control group, where the AChR concentration was 18.4 ± 2 fmol/mg protein; (2) from 3.9 ± 0.3 to 5.1 ± 0.1 in the mobile dTC-infused group (AChRs = 48.6 ± 7); (3) from 3.8 ± 0.1 to 5.5 ± 0.3 in the immobilized saline-treated group (AChRs = 107.4 ± 14); and (4) from 3.8 ± 0.1 to 6.3 ± 0.2 in the immobilized-dTC-treated group (AChRs = 185.5 ± 23). There was a significant positive correlation between maximal change in blood potassium concentration and the respective AChR concentration in the gastrocnemius of the same animal (r = 0.81, P < 0.01).

Conclusions: Subtherapeutic (subparalytic) doses of chronic infusion of dTC (with no immobilization) or immobilization alone (with no dTC) independently increased number of AChRs. The infusion of dTC with immobilization caused the greatest upregulation of AChRs. The magnitude of the increase in blood potassium to SCH was directly dependent on AChR number. This study shows direct evidence and confirms previous speculation that AChR number plays an important role in the magnitude of the hyperkalemic response to SCH. Premising this represents an appropriate model for patients who are immobilized and/or receiving nondepolarizing muscle relaxants for prolonged periods, exaggerated blood potassium responses to SCH are possible when either of both of these perturbations are present in patients. (Key words: Complications; hyperkalemia; immobilization. Neuromuscular relaxants: d-tubocurarine; nondepolarizing; succinylcholine. Receptor: acetylcholine; nicotinic.)

HYPERKALEMIC cardiac arrest after succinylcholine (SCH) administration has been reported in patients after lower or upper motor neuron injuries, muscle trauma, burns, or radiation injuries.1–3 An upregulation (increase) of nicotinic acetylcholine receptors (AChRs) on the muscle membrane has been associated with the increased sensitivity and hyperkalemia to SCH. Hyperkalemic cardiac arrest after SCH in critically ill intensive care patients without the aforementioned conditions also has been reported.4–8 Chronic administration of nondepolarizing muscle relaxants (NDMRs), such as d-tubocurarine (dTC), with subsequent long periods of immobi-
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Immobilization has been implicated in the altered sensitivity to Sch, but this hypothesis has not been tested. Immobilization and disuse atrophy of muscle by pinning of joints has been reported to proliferate ACHR.9,10 In other studies, although ACHRs were not quantitated, immobilization by application of a plaster cast has been shown to cause moderate hyperkalemia to Sch and resistance to NDMR.11-13 The magnitude of the increase in ACHRs associated with this model of immobilization and its relationship to Sch-induced hyperkalemia (or resistance to NDMR) has not been evaluated and may be of clinical importance.

Prolonged paralysis caused by antagonists (inhibitors) of ACHR, such as α-bungarotoxin and NDMR, has been shown to upregulate ACHR on the muscle membrane.14-16 These studies, however, did not differentiate the effects of immobilization versus ACHR inhibition on these receptor changes. Chronic inhibition of any receptor, even in the absence of demonstrable functional changes, often can upregulate receptor number itself.17,18 This has been confirmed, relative to skeletal muscle ACHR, where chronic subclinical inhibition of neuromuscular transmission or of ACHR by drugs, such as dTC and phenytoin, caused an increase of these receptors, even in the absence of immobilization.19-21 These studies also have shown that the drug-induced upregulation of ACHR can be associated with resistance to NDMR.16,19,20 The objectives of the current study were to characterize the contributory roles of chronic administration of an NDMR such as dTC and of immobilization, alone and in combination, on ACHR number and its relationship to blood potassium changes after the administration of Sch.

Methods

All procedures in this study conformed to Institutional and National Institutes of Health animal care guidelines. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA), weighing 375-450 g were used. To discriminate the effects of chronic inhibition of ACHR from that of immobilization on ACHR upregulation, rats were randomly assigned to one of four groups. The most extensively used and studied pharmacologic probe of the neuromuscular junction, dTC (Sigma Chemical, St. Louis, MO), was used as the competitive antagonist of ACHR. The four groups consisted of saline-treated mobile control (group 1, n = 12), dTC-treated mobile (group 2, n = 22), saline-treated immobilized (group 3, n = 10), and dTC-treated immobilized (group 4, n = 19) groups.

The animals in all groups received continuous, chronic infusions of saline (groups 1 and 3) or dTC (groups 2 and 4) using Alzet osmotic pumps (Alza, Palo Alto, CA). The animals were anesthetized with 40-50 mg/kg of intraperitoneal pentobarbital sodium (Abbott Labs, Chicago, IL) with subsequent doses administered as required. After the animals were shaved on the dorsum, the skin was prepared with iodine solution. A 1.5-2-cm transverse incision was made in the intercostal area of the back, and a subcutaneous pocket was constructed by blunt dissection. In each of the animals, an osmotic pump, filled with saline or dTC, was inserted into the subcutaneous pocket. The osmotic pumps (model 2ML4) delivered a constant infusion of 2.38 ± 0.07 μl/h. Groups 1 and 3 received this volume as saline only. Groups 2 and 4 received the same volume, via the pump, but the saline was spiked with dTC, delivering a dose of 25 μg·kg⁻¹·h⁻¹. Preliminary experiments revealed that exposure to this dose of dTC did not result in paralysis and permitted animals to feed, drink, and move freely about their cages. After implantation of osmotic pumps, the incisions were closed with 2-0 silk, antibiotic ointment was applied to the wound, and the rats were returned to the animal care area. Animals received the same postoperative care, including food and water ad libitum and exposure to alternating light and dark environment closed cycles.

Immobilization

Animals in groups 3 and 4 underwent bilateral hind-limb immobilization by means of a plaster cast where both knees and ankles were immobilized at 70° and 90° respectively. The casts were reinforced with fiberglass to reduce possible damage from chewing. Casts were changed, as needed, every 4-10 days under light pentobarbital anesthesia (25-30 mg/kg, intraperitoneal). Each animal, regardless of group, received an identical dose of anesthetic on the days the casts were changed. Care was taken to ensure that all animals with bilateral hind-limb immobilization had easy access to food and water. Animals that removed their casts were not included in the study. The infusions and/or casts were maintained for 28 days.
Analysis of Plasma d-Tubocurarine Concentrations

On day 28, the animals were anesthetized as described earlier and 0.5 ml blood was withdrawn from the lateral tail vein for determination of plasma dTC concentration by high-perfusion liquid chromatography as described previously. Blood withdrawn was replaced with three times the volume of normal saline. After blood sampling, the osmotic infusion pump was removed from the interscapular area, the incision was closed, and the animal was returned to the animal care facility. The lower detection limit of the assay of dTC was 25 ng/ml in plasma. Based on preliminary studies, a concentration ≥10 μg/ml was considered therapeutic for upregulation of AChRs; animals having plasma dTC concentrations less than 10 μg/ml were not included in the study or data analysis.

Blood Potassium Response to Succinylcholine

The animals were reanesthetized with pentobarbital, as described previously, during the interval of 24–48 h after removal of osmotic pumps. Preliminary studies (n = 6) revealed that approximately 24–48 h after removal of the osmotic pumps, the dTC levels were undetectable in plasma. A tracheostomy was performed, and the animal’s lungs were ventilated with room air with the use of a Harvard Ventilator to maintain physiologic venous blood gases. Venous access for drug (SCH) administration was secured via the jugular vein. Rectal temperature was monitored and maintained at 36–37°C with the use of heat lamps. A laparotomy was then performed to cannulate the inferior vena cava just above its formation from the iliac veins, for sampling of venous blood returning from the two hind limbs. This procedure thus ensured sampling of blood returning from the lower limbs only. After obtaining a baseline venous blood sample (0.5 ml) from the inferior vena cava, 5 mg/kg SCH was administered as a bolus, through the jugular vein and flushed in with approximately 0.25 ml saline. Blood samples were obtained from the inferior vena cava at 1, 3, 5, 7, and 10 min after SCH administration for determination of potassium concentration with an Astra ion-selective electrode (Beckmann Instruments, Brea, CA).

Acetylcholine Receptor Assay

At the end of the potassium-response study, the animals were killed with an overdose of pentobarbital and the left gastrocnemius muscle was removed for determination of AChR concentrations. The muscle was excised, washed several times in saline, chopped quickly, and frozen at −70°C until quantitation of AChR concentrations. 125I-α-bungarotoxin (specific activity 16.8 μCi/μg, New England Nuclear, Boston, MA), which binds specifically and irreversibly to the AChRs, was used for their quantitation. The assay procedure has been reported in detail previously. On the day of the assay, the muscles were thawed and homogenized for 1 min in 4 volumes of 0.01 M potassium phosphate buffer, pH 7.4, containing 1 mm EDTA, 2 mm benzamidine hydrochloride, 0.1 mm phenylmethylsulfonyl fluoride, 0.5 mg/ml bacitracin, and 0.02% (w/v) sodium azide at 4°C. The homogenate was centrifuged at 20,000g for 30 min at 4°C. The precipitates were resuspended and homogenized for 1 min in the same buffer containing an additional 2% (vol/vol) Triton X-100 (Sigma Chemical, St Louis, MO), a detergent that extracts the AChRs. The extraction procedure was continued overnight, on a shaker, in a cold room. The solution was centrifuged at 20,000g for 50 min at 4°C, the supernatant was recovered and stored at −70°C. Triplicate samples of crude muscle extract were incubated with 2.5 nm 125I-α-bungarotoxin in the 2% Triton-buffer for 90 min at room temperature. Excess 125I-α-bungarotoxin was separated from toxin-bound AChR complex using polyethyleneimine, pretested Whatman GF/B glass fiber filters by vacuum filtration. Nonspecific binding of α-BTX was detected by preincubation, with excess unlabeled 1 μM of α-BTX. The protein concentration of muscle extract was assayed according to the Hartree method. Based on the molecular weight and specific activity of α-BTX, counts per minute and disintegrations per minute, the receptor concentrations were calculated and was expressed as femtomoles per milligram of protein.

Statistical Analysis

Differences between groups were tested by one-way analysis of variance, followed by t tests with Dunn’s corrections for multiple comparisons with overall α = 0.05. The additivity of the effect of dTC and immobilization was tested by two-way analysis of variance. For receptor analysis, the concentrations were converted to log scale because it stabilized the variances in all groups and improved the fit of the model. The difference between pre-SCH and the maximum increase in potassium concentration was correlated with AChR number, both before and after subtracting the mean for each group from each observation in that group.
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Table 1. Blood K⁺ Concentrations before and after Sch (mEq/L)

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>+1</th>
<th>+3</th>
<th>+5</th>
<th>+7</th>
<th>+10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n = 12) (saline alone)</td>
<td>3.9 (0.04)</td>
<td>4.1 (0.03)</td>
<td>4.3 (0.1)</td>
<td>4.5 (0.1)</td>
<td>4.4 (0.1)</td>
<td>4.2 (0.1)</td>
</tr>
<tr>
<td>2 (n = 12) (DTC alone)</td>
<td>3.9 (0.03)</td>
<td>4.5 (0.1)</td>
<td>4.8 (0.1)</td>
<td>5.1 (0.1)</td>
<td>5.2 (0.1)</td>
<td>5.2 (0.1)</td>
</tr>
<tr>
<td>3 (n = 10) (immobilized alone)</td>
<td>3.8 (0.1)</td>
<td>4.8 (0.1)</td>
<td>5.4 (0.2)</td>
<td>5.5 (0.3)</td>
<td>5.3 (0.2)</td>
<td>5.3 (0.3)</td>
</tr>
<tr>
<td>4 (n = 10) (immobilized + DTC)</td>
<td>3.8 (0.1)</td>
<td>5.3 (0.2)</td>
<td>6.1 (0.1)</td>
<td>6.3 (0.2)</td>
<td>6.2 (0.2)</td>
<td>5.9 (0.2)</td>
</tr>
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</table>

Values are mean SEM.

by Pearson's correlation coefficient. P < 0.05 was considered significant. Values are expressed as mean ± SEM.

Results

Changes in Body Weight and d-Tubocurarine Concentrations

Eight animals in group 2 had plasma dTC concentrations ≤10 μg/ml and an additional two died before 28 days had passed. Group 4 had attrition of nine animals because of dTC levels ≤10 ng/ml, falling off of plaster casts and/or death before testing of the potassium response to Sch. Thus, total number of animals tested for potassium response and AChR concentrations consisted of n = 12 in each of groups 1 and 2 and n = 10 in groups 3 and 4. Two animals in group 4 died 5–7 min after the administration of Sch. In these cases, the cause of death is unknown and may or may not have been related to hyperkalemia. Animals exposed to dTC with no hind-limb immobilization could stand on their hind limbs and were able to eat, drink, and move about their cages freely. The weight gains in groups 1 and 2 at the end of 28 days were 130 ± 7 and 152 ± 6 g, respectively, confirming that the latter group's ability to feed was not unduly affected by the dTC infusion. Although care was taken to ensure that all animals had free access to food and water, the weight gains in groups 3 and 4 at 28 days were only 72 ± 6 and 68 ± 7 g, respectively, which were not significantly different from each other, but were significantly (P < 0.01) less than groups 1 and 2. The dTC concentrations, measured after 28 days of DTC infusion were 0.32 ± 0.09 μg/ml and 0.36 ± 0.08 μg/ml for groups 2 and 4, respectively.

Blood Potassium Concentrations Before and After Succinylcholine

The potassium concentrations for each group recorded at baseline (before Sch) and at 1, 3, 5, 7, and 10 min after Sch are shown in table 1. The greatest increase in blood potassium concentration was observed 5 min after Sch. The mean change in potassium concentrations, calculated from the highest measured blood potassium concentration minus the baseline concentration for each animal also is shown in figure 1A. In group 1, the increase was 0.6 ± 0.1 mEq/L. The maximal change in potassium in group 2 was 1.1 ± 0.2 mEq/L, which was significantly greater than group 1, as was the potassium response in group 3 relative to group 2. The greatest potassium response to Sch occurred in group 4 (dTC + immobilization), where the change

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was $2.5 \pm 0.2$ mEq/l above baseline and was significantly higher than that of group 3 (saline + immobilization) where the potassium change after SCh was $1.7 \pm 0.3$ mEq/l ($P < 0.01$). That is, groups 2, 3, and 4 had significant increases in potassium after SCh when compared to group 1 ($P < 0.0001$).

**Acetylcholine Receptor Concentrations and Potassium Levels**

All experimental groups showed a significant increase in the concentration of AChRs when compared with group 1 (fig. 1B; $P < 0.0001$). Group 4 showed the highest AChR number ($184 \pm 23$ fmol/mg protein) and was significantly greater than group 3 (saline + immobilization, $107 \pm 14$ fmol/mg protein), which was significantly greater than group 2 ($P < 0.05$). The effects of dTc and immobilization on AChRs were additive, as observed also for potassium concentrations. The correlation between each maximal change in potassium concentration to its respective log AChR concentration at the left gastrocnemius muscle combining all groups, was 0.81, $R^2 = 0.66$ ($P < 0.01$); that is, 66% of the variability in potassium was explained by changes in AChRs. After removing the treatment effects by subtracting the mean for each group from each observation in that group, for both variables, the peak potassium change and log AChR were still significantly correlated ($r = 0.46$, $P = 0.0015$).

**Discussion**

A well-established pharmacologic doctrine is that, in most instances, upregulation of a receptor is associated with resistance (hyporesponsivity) to competitive antagonists (e.g., NDMRs). Relative to AChRs and neuromuscular relaxants, this relationship has been confirmed in a number of studies where upregulation of AChRs was associated with increased requirements for NDMRs. Conditions in which this association has been established include burns, denervation, and chronic administration of muscle relaxant or anticonvulsant therapy. In these same conditions, there are numerous reports of increased sensitivity of the neuromuscular junction to agonists such as acetylcholine or SCh; that is, dose-response curves to these agonists were shifted to the left. Although exceptions do occur, a corollary of this left-ward shift to agonists is the hyperkalemic response to the agonist, succinylcholine, in some of the conditions described earlier. Nonetheless, the relationship of the potassium response to actual receptor number has not been examined. All previous studies have either looked at AChR distribution and sensitivity to iontophoretically applied acetylcholine or examined SCh-induced potassium response in conditions where there was a priori evidence for upregulation of AChRs. Thus, the relationship of potassium release after SCh to AChR number was unknown.

The current study evaluated the role and importance of competitive antagonism of the AChR (induced by dTc) and of immobilization (induced by application of bilateral hind-limb plaster cast), alone and in combination on AChR number, and elucidated further, the relationship of the potassium release into plasma after SCh to AChR concentrations. The salient findings of this study are (1) that infusion of dTc upregulates AChRs even in the absence of immobilization and confirms our previous observations; (2) that immobilization by plaster cast can also upregulate AChRs in the absence of denervation, and confirms observations made in other models of immobilization; (3) that AChR upregulation induced by immobilization can be enhanced by concurrent administration of dTc; (4) that each of these perturbations also caused an exaggeration of the potassium release after SCh; and (5) that among animals within the same treatment group, the highest potassium concentrations were correlated with higher AChR concentration, a relationship that was also observed when all groups were pooled together (fig. 2).

The cause and effect relationship between AChR concentration and potassium response is emphasized by the significant association between these variables, even after removing the treatment effects by subtracting the mean for each group from each observation in that group, for both variables. This relationship was also evident when all groups were pooled together. The dTc infusion, immobilization, or their combination did not, however, increase the receptor numbers to that level seen with denervation. In the current studies, the AChR concentrations associated with each of these perturbations, did not exceed 200 fmol/mg protein. In contrast, our studies have documented that after partial denervation the AChR proliferation is in excess of 1000 fmol/mg protein. The molecular mechanism by which each of the perturbations that we studied upregulated AChRs has not been elucidated in this study. The physiologic state of immobilization contrasts with that of denervation syndromes in that there is no direct damage to cord or nerve roots. The muscle fibers, AChE, and the nerve terminals, are all intact.
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![Graph showing peak change in plasma K⁺ levels against log AChR concentration](image)

**Fig. 2.** Regression analysis of peak change in potassium concentrations in each animal (relative to baseline) after succinylcholine to acetylcholine receptor concentrations in the gastrocnemius muscle of the same animal. Each group is denoted by a different symbol with key to each group indicated in the figure. There was a significant positive correlation between peak potassium change and acetylcholine receptor concentration ($R^2 = 0.66$, $r = 0.81$, $P < 0.01$).

After immobilization, do remain innervated, demonstrated by the presence of miniature end-plate potentials and the brisk contraction when the nerve supplying the immobilized muscle is stimulated.¹⁰

We speculate that the upregulation of AChRs in the plaster-cast model of immobilization (with no nerve damage) is probably related to both transcriptionally and translationally mediated mechanisms. Decreased activity is associated with increased transcripts (mRNA levels) of AChR subunits and direct electrical stimulation of denervated muscles attenuates the upregulation of these receptors and their transcripts.¹⁰ ¹³ Additionally, increased cyclic adenosine monophosphate activity or cortisol levels have been shown to cause increased assembly and surface expression of AChRs by a posttranscriptional (translational) mechanism; that is, receptor changes were induced in the absence of changes in transcripts.³²⁻³⁵ Immobilization itself results in a stress response with release of catecholamines (increased cyclic adenosine monophosphate activity) and cortisol for prolonged periods.³⁶⁻³⁷ This humoral response to immobilization, stress, which may increase assembly and expression of cell surface AChRs, may have been an alternative or additional mechanism for the increase in receptors.

The molecular mechanism of upregulation of AChRs by chronic infusions of dTC (in the absence of immobilization) also has not been elucidated in this or other studies. The studies of Chang et al.,¹⁴ Berg and Hall,¹⁷ and Dodson et al.,¹⁰ although reporting an increase of AChRs with paralysis produced by muscle relaxants, did not elucidate the factors contributing to these changes. Several models of drug-induced immobilization (paralysis), however, have been studied by others. These studies applied toxins (tetradotoxin, bungarotoxin or botulinum toxin) to decrease nerve or muscle function with immobilization or paralysis, and quantitated the receptor protein and its subunit transcripts.³⁸ ³⁹ These toxins, including bungarotoxin, an AChR antagonist, caused the upregulation of AChRs probably by increased transcriptional activity as all of its subunit transcripts, including $\alpha$, $\beta$, $\delta$, $\epsilon$, and $\gamma$-subunits, were increased. It is important to note that, despite the absence of motor denervation, increased expression of the $\gamma$-subunit mRNAs and the receptor protein incorporating it, was observed. Typically, expression of $\gamma$-subunit is seen in denervation-induced paralysis.

In our study, there was no apparent evidence of muscle paralysis with the dTC infusion. Whether the subclinical, subtherapeutic doses of dTC cause an upregulation of AChRs by a transcriptional mechanism, is currently being investigated. In burn injury, increased receptor number at sites distant from the burn has been observed in the absence of increased mRNA levels.⁴⁰ Thus, the increase in AChRs after burns at sites distant from the area of injury is probably related to a posttranscriptional mechanism.⁴⁰ A similar posttranscriptional mechanism may be operative in our subparalytic model.

Whether administration of paralytic doses of NMDRs will result in gene-mediated upregulation of AChR subunits including expression of the immature receptors containing $\gamma$-subunits (similar to that seen with application of $\alpha$-bungarotoxin), will be important.
to assess and may be particularly relevant to the muscle weakness associated with chronic infusions of NDMRs. 5, 6, 42

The clinical implications of this study are as follows: critically ill patients, especially those in intensive care units, are immobilized for prolonged periods. Furthermore, these patients also receive therapeutic doses of NDMRs for various indications. It is evident from the current study that both the infusion of NDMR, typified by dTC and/or immobilization, will result in the upregulation of AChRs and that upregulation of AChRs is associated with increased potassium release with succinylcholine even after holding treatments constant. The combined effects of both will result in further exaggeration of this upregulation of AChRs. The use of ScH for changing endotracheal tubes or for reintubation is common in the intensive care unit because of its rapid action and short duration. Cardiac arrest with lethal potassium concentrations has been reported in some of these instances. 5-6 It is likely, based on this study, that immobilization and chronic neuromuscular blockade are important factors contributing to the upregulation of AChRs and to the cardiac arrest associated with the use of ScH. For this reason, it may be advisable to avoid depolarizing relaxants in patients previously immobilized and/or exposed to extended use of NDMRs. Pretreatment with NDMRs to attenuate the potassium responses to ScH is futile because the doses of NDMRs necessary for this are high, and often the subsequent potassium responses are unpredictable. 1, 2, 4 The onset and duration of these changes in AChRs after chronic immobilization and NDMRs and the role of these changes in critical illness-induced muscle weakness are unknown and need to be evaluated further.

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