

Immobilization with Atrophy Induces *De Novo* Expression of Neuronal Nicotinic $\alpha 7$ Acetylcholine Receptors in Muscle Contributing to Neurotransmission

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ABSTRACT

Background: Mature acetylcholine receptor (AChR) isoform normally mediates muscle contraction. The hypothesis that $\alpha 7$ AChRs up-regulate during immobilization and contribute to neurotransmission was tested pharmacologically using specific blockers to mature (waglerin-1), immature (αA -OIVA), and $\alpha 7$ AChRs (methyllycaconitine), and nonspecific muscle AChR antagonist, α -bungarotoxin.

Methods: Mice were immobilized; contralateral limbs were controls. Fourteen days later, anesthetized mice were mechanically ventilated. Nerve-stimulated tibialis muscle contractions on both sides were recorded, and blockers enumerated above sequentially administered *via* jugular vein. Data are mean \pm standard error.

Results: Immobilization ($N = 7$) induced tibialis muscle atrophy (40.6 ± 2.8 vs. 52.1 ± 2.0 mg; $P < 0.01$) and decrease of twitch tension (34.8 ± 1.1 vs. 42.9 ± 1.5 g; $P < 0.01$). Waglerin-1 (0.3 ± 0.05 μ g/g) significantly ($P = 0.001$; $N = 9$) depressed twitch tension on contralateral ($\geq 97\%$) *versus* immobilized side (approximately 45%). Additional waglerin-1 (total dose 1.06 ± 0.12 μ g/g or approximately $15.0 \times ED_{50}$ in normals) could not depress twitch of 80% or greater on immobilized side. Immature AChR blocker, αA -OIVA (17.0 ± 0.25 μ g/g) did not change tension bilaterally. Administration of α -bungarotoxin ($N = 4$) or methyllycaconitine ($N = 3$) caused 96% or greater suppression of the remaining twitch tension on immobilized side. Methyllycaconitine, administered first ($N = 3$), caused equipotent inhibition by waglerin-1 on both sides. Protein expression of $\alpha 7$ AChRs was significantly ($N = 3$; $P < 0.01$) increased on the immobilized side.

Conclusions: Ineffectiveness of waglerin-1 suggests that the twitch tension during immobilization is maintained by receptors other than mature AChRs. Because αA -OIVA caused no neuromuscular changes, it can be concluded that immature AChRs contribute minimally to neurotransmission. During immobilization approximately 20% of twitch tension is maintained by up-regulation of α -bungarotoxin- and methyllycaconitine-sensitive $\alpha 7$ AChRs. (**ANESTHESIOLOGY 2014; 120:76-85**)

NICOTINIC acetylcholine receptors (AChRs) on the skeletal muscle membrane are pivotal for neurotransmission and muscle contraction.^{1,2} Typically, two types of muscle AChRs, the immature or fetal AChRs containing $2\alpha 1\beta 1\delta\gamma$ subunits, and the mature AChRs consisting of $2\alpha 1\beta 1\delta\epsilon$ subunits have been described.¹⁻⁵ In the normal innervated muscle, the mature AChRs are present only in the junctional area, and are involved in neurotransmission. When there is deprivation of neural influence or activity (*e.g.*, fetus or denervation), the γ subunit containing immature AChRs are up-regulated and expressed throughout the muscle membrane.¹⁻⁵ Neuronal nicotinic AChRs containing five homomeric $\alpha 7$ -subunits, previously described only in the central nervous system,

What We Already Know about This Topic

- Immature nicotinic acetylcholine receptors (AChRs) are up-regulated in skeletal muscle after denervation or immobilization, but their roles in neurotransmission are unknown
- The effect of unilateral immobilization on AChR expression and function was studied in a mouse model using selective antagonists of various AChR subtypes

What This Article Tells Us That Is New

- After immobilization, immature AChRs did not contribute to neurotransmission; however, $\alpha 7$ AChRs were up-regulated and also contributed to neuromuscular transmission
- Resistance to clinically used muscle relaxants seen with immobilization might be related to functional expression of insensitive $\alpha 7$ AChRs

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have more recently been described in skeletal muscle after denervation only.^{6,7}

In some pathologic states, despite the presence of innervation, up-regulation of the immature AChRs occurs.^{3–5} For example, several studies in the past 3 decades using varied models of immobilization have shown that disuse of muscle leads to muscle atrophy, and *de novo* expression of immature AChRs throughout the muscle membrane, despite the presence of continued innervation.^{3–5,8–11} The up-regulation of immature AChRs during immobilization has been documented using electrophysiology, ligand binding, *in situ* hybridization, or polymerase chain reaction techniques.^{2,8–12} This up-regulation of immature AChRs with immobilization occurs mostly in the extrajunctional area,^{8,11,12} although the junctional-area expression has been documented by *in situ* hybridization.¹² The contribution of immature AChRs to neurotransmission during immobilization is unknown. It has been assumed, however, that the expression of the immature AChRs in the junctional area contributes to the resistance to nondepolarizing muscle relaxants during immobilization.^{3–5,9–11} Although the immobilized muscle behaves like denervated muscle in some aspects (*e.g.*, muscle wasting and up-regulation of immature AChRs), whether $\alpha 7$ AChR expression also occurs after immobilization, as in denervation, has never been tested.

Cobra snake (*Bungarus multicinctus*) venom, α -bungarotoxin (α BTX), can bind irreversibly to all muscle AChRs including $\alpha 7$ AChRs to block acetylcholine-induced currents or neurotransmission.^{1,2} Thus α BTX, like clinically used muscle relaxants (pancuronium, atracurium), has no specificity for the three AChR isoforms expressed in muscle.^{1,2,8} A snake toxin from the viper, *Trimeresurus wagleri*, called waglerin-1, has been described and binds with high-affinity and high-selectivity only to mature AChRs *in vitro* in oocytes and *in vivo* in mice.^{13–15} More recently, toxins from marine cone snails, *Conus obscurus* and *Conus pergrandis*, termed α A-OIVA have been characterized and inhibit only the fetal (immature) AChRs with high affinity and unprecedented specificity both *in vitro* in oocytes and *ex vivo* in rodents.^{14,16–18} Methyllycaconitine is a specific $\alpha 7$ AChR antagonist derived from *Delphinium (larkspur)* plant. Its specificity for the $\alpha 7$ AChRs has been described both *in vivo* and *in vitro*.^{19–21}

In this study using mechanomyographic techniques together with waglerin-1, α A-OIVA, and methyllycaconitine, as specific antagonist ligands to the mature, immature,

and $\alpha 7$ AChRs, respectively, and with α BTX, we tested the hypothesis that $\alpha 7$ AChRs are up-regulated in muscle after immobilization and contribute to neurotransmission, whereas the up-regulated immature AChRs contribute minimally to neurotransmission. In addition to *in vivo* pharmacological methods, the presence of $\alpha 7$ AChR protein on the muscle membrane after disuse was also confirmed biochemically by the use of immunoblot (Western blot).

Materials and Methods

Animals

The study was approved by the Subcommittee on Research Animal Care at the Massachusetts General Hospital, Boston, Massachusetts. Adult male C57BL/6J mice (Jackson Labs, Bar Harbor, ME), weighing 25–30 g, were used for the study. The mice were housed under a 12-h light/dark cycle with food and water available *ad libitum* and were allowed to accommodate to the standard conditions of our facility for at least a week.

Surgical Procedures

The pinning-immobilization model, previously described and used in many studies, was used for the current studies.^{8–11} After 1 week of acclimatization, immobilization procedure was performed. The mice were anesthetized with pentobarbital (60–70 mg/kg intraperitoneal). The knee and ankle joints were immobilized by insertion of a 25-gauge hypodermic needle through the proximal tibia into the distal femur to produce 90° flexion at the knee, and a 27-gauge needle through the calcaneus into the distal tibia to fix the ankle joint at 90°. The sham-immobilized limb was subjected to the same manipulations, including boring a hole through the joints, but the pins were not maintained to immobilize the joint. On the basis of our previous reports, it was found that sham-immobilization of the contralateral leg does not alter muscle function, morphology, AChR number, or muscle weight compared with unimmobilized limbs of naïve rodents.^{10,11} A more recent study by us in mice again confirmed that the contralateral side does not differ from that in unimmobilized naïve animals.²² In the current study, therefore, the contralateral sham-immobilized hind limb served as the control. After recovery from anesthesia, the animals were returned to their cages.

Neuromuscular Function Studies

To characterize the role of each AChR isoform to neurotransmission, nerve-evoked tibialis muscle tension responses were recorded at 14 days after immobilization. The mice were anesthetized with pentobarbital (60–70 mg/kg intraperitoneal) and tracheostomy was performed for mechanical ventilation with air at 140–150 breaths/min with a tidal volume of 6–8 ml/kg (MiniVent Type 845; Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany). The jugular vein was cannulated for fluid and drug administration. Adequate depth of anesthesia was confirmed by

Peptide Core Facility, Massachusetts General Hospital (A.K.); the Signal Transduction Laboratory, Massachusetts General Hospital, and Harvard Medical School (M.K.), the Clinical and Biochemical Pharmacology Laboratory, Massachusetts General Hospital, Shriners Hospitals for Children®, and Harvard Medical School (J.A.J.M.). Current positions: Sang-gye Paik Hospital, College of Medicine, In-Je University, Seoul, South Korea (S.L.), Department of Anesthesiology, Asan Medical Center, University of Ulsan, Seoul, South Korea (H.-s.Y.), Asahikawa University, Asahikawa, Japan (T.S.).

the absence of the withdrawal response to toe clamping. Anesthesia was maintained with supplemental intermittent doses of pentobarbital 10–20 mg/kg intraperitoneal, empirically administered every 15–20 min. The body temperature was monitored using a rectal thermistor and maintained at 35.5°–37°C with a heat lamp.

Neuromuscular transmission was monitored by evoked mechanomyography by using a peripheral nerve stimulator (NS252; Fisher & Paykel Health Care, Irvine, CA) along with a Grass Force transducer and software (Grass Instruments, Quincy, MA). With the mice in dorsal recumbency, the tendon of insertion of the tibialis muscle was surgically exposed on each side and individually attached to separate grass FT03 force displacement transducers. The sciatic nerve was exposed at its exit from the lumbosacral plexus at the thigh and tied with ligatures for indirect nerve stimulation of the muscles. Distal to the ligatures, stimulation electrodes were attached for nerve-mediated stimulation of the tibialis muscle. The knee was rigidly stabilized with a clamp to prevent limb movement during nerve stimulation. Baseline tensions of 10 g, which yielded optimal evoked tensions, were applied on the immobilized and sham-immobilized tibialis muscles. The sciatic-nerve-evoked tensions of the respective tibialis muscles that were calibrated in grams of force were recorded *via* a Grass P122 amplifier and displayed using the Grass Polyview Software (Grass Instruments). Supramaximal electrical stimuli of 0.2 ms duration were applied to the sciatic nerve at 2 Hz for 2 s (train-of-four pattern) every 30 s using a Grass S88 stimulator and SIU5 stimulus isolation units (Grass Instruments). In view of the fact that the main focus of the experiments were to characterize the expression of the immature and $\alpha 7$ AChRs and their function in neurotransmission, detailed evaluation of the muscle function (*e.g.*, tetanic contraction and fade) was not performed. Furthermore, repetitive tetanic stimulation during the cumulative administration of the AChR antagonists will result in fatigue of muscle.

Study of the Role of Mature AChRs in Neurotransmission

The sciatic nerve-tibialis muscle preparation *in vivo* was stabilized for at least 30 min, before the administration of specific AChR antagonists. In the initial set of experiments, waglerin-1, the specific ligand for mature AChRs,^{13–15} synthesized by the Peptide Core Facility, at Massachusetts General Hospital, was administered intravenously and the twitch responses noted for each cumulative dose. L.D.₅₀ of waglerin-1 in mice is approximately 0.33 mg/kg intraperitoneally (95% CI, 0.30–0.36).²³ An initial bolus dose of 5 μ g of waglerin-1 was administered intravenously followed by increments of 2 μ g until the first twitch (T_1) in the train-of-four stimulations decreased to 75% or greater of baseline on both sides. Each incremental dose of waglerin-1 (2 μ g) was given only when the previous dose had produced maximal effect, indicated by three equal consecutive T_1 twitches in both muscles or an increasing T_1 response in either muscle. The interval between doses was 3–4 min. In

view of the poor response to waglerin-1 on the immobilized side, additional doses were given until twitch height did not change with repetitive doses.

Study of the Role of Immature AChRs in Neurotransmission

The second phase of our experiment was the study of the role of immature AChRs in neurotransmission during immobilization. The α -conotoxin, α A-OIVA, a highly selective blocker of the immature AChR (synthesized by Peptide Core Facility, Massachusetts General Hospital), was used. Previous studies have tested its potency to block immature AChRs in oocytes and its efficacy to block acetylcholine-induced currents in the immature AChR in the rat *ex vivo*.^{14,16–18} The L.D.₅₀ of α A-OIVA *in vivo* is unknown because immature AChRs are not normally expressed. This ligand blocks mouse fetal muscle AChRs expressed in oocytes (IC₅₀ of 0.51 nM or 0.94 ng/ml) with approximately 600-fold greater affinity than for the adult mature muscle AChRs.^{14,16,17} The IC₅₀ for *ex vivo* blocking of fetal receptors expressed in denervated muscle of rodents is 1 μ M.¹⁸

When the twitch responses did not change with continued administration of waglerin-1 in the studies described above, cumulative doses of α A-OIVA were administered intravenously every 3–4 min while observing twitch responses, alternating with waglerin-1, if twitch tension was recovering. When the twitch tension did not change with α A-OIVA, α BTX (which blocks mature, immature and $\alpha 7$ AChRs) was administered (0.165 μ g/g, each time) intravenously every 3–5 min until 97% or greater twitch depression.

Study of the Role of $\alpha 7$ AChRs in Neurotransmission

The nerve-muscle preparation was the same as described previously. In a new group of mice, after stabilization of twitch, waglerin-1 and α A-OIVA were administered until twitch responses were unchanged after these toxins. At this time methyllycaconitine, derived from delphinium-larkspurs alkaloid (Sigma, St. Louis, MO), a specific antagonist of $\alpha 7$ AChRs,^{19–21} was administered in incremental doses.

We then posited that if $\alpha 7$ AChRs are the cause of the resistance to block by waglerin-1, if one preemptively blocks the $\alpha 7$ AChRs, then the response to waglerin-1 should be equal on the immobilized and contralateral sides. Therefore, in a separate set of animals, after stabilization of the nerve-evoked muscle tension responses, methyllycaconitine (50 μ g) was administered as a bolus to block the $\alpha 7$ AChRs in muscle on both sides. After a period of 10 min, α A-OIVA was administered, as performed previously, and changes in twitch tension noted. When twitch tension was unchanged, waglerin-1 was administered.

Immunoblot for Protein Expression of $\alpha 7$ AChRs on Muscle Membrane

After the termination of the functional studies on methyllycaconitine, the tibialis muscle was harvested and stored

at -80°C . At a later time, the muscle samples were thawed, powdered in liquid nitrogen, and homogenized in homogenization buffer (Sigma) containing protease inhibitor (Roche, San Francisco, CA) as described previously.²⁴ The samples were centrifuged, and aliquots of the supernatant containing equal amounts of protein (by Bradford Assay) were subjected to SDS-PAGE. Immunoblotting was performed as described previously.²⁵ Equal amounts of protein (40 μg) per lane were subjected to NuPAGE and then blotted onto nitrocellulose membrane (Invitrogen, Carlsbad, CA). The membrane was blocked by 5% nonfat dry milk. Anti- $\alpha 7\text{AChR}$ (dilution 1:1,000), antiglyceraldehyde 3-phosphate dehydrogenase (1:20,000; Abcam, Cambridge, MA) were used as primary antibodies. The membranes were incubated in horseradish peroxidase-conjugated goat anti-mouse secondary antibody for 30 min (dilution 1:5,000). The specific proteins were detected by exposing the membrane to Kodak X-Omat films (Hyglo Quick Spray; Denville Scientific, Inc., Metuchen, NJ).

Statistical Analyses

Values are expressed as mean \pm SEM. All doses were converted to $\mu\text{g/g}$ body weight. The differences of baseline twitch heights, weights of tibialis muscles, and specific twitch tensions between immobilized and contralateral sides were analyzed by paired-sample t test (two-tailed, not equal-variance assumption). The changes in percent twitch depression of first twitch tension (T_1) relative to baseline after administration of the study drugs (waglerin-1, $\alpha\text{A-OIVA}$, αBTX , and methyllycaconitine), and the cumulative doses of the study drugs at each measure point between the two sides were compared using repeated measures ANOVA and multiple comparative tests with Bonferroni correction. To determine effective dose of waglerin-1 for 50 and 95% twitch depression (ED_{50} , ED_{95}) on each side, the percent twitch depression of the first twitch of train-of-four (T_1) relative to baseline was transformed to logit scale and plotted against the logarithm of the cumulative dose, and then linear regression analyses were performed. Comparison between ED_{50} and ED_{95} values for each side were made using unpaired Student two-tailed t test with Welch corrections. Values were assumed to be significant if the P value was less than 0.05. All statistical

tests were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA).

Results

Stability and Consistency of Model

Our initial set of experiments were to characterize the muscle mass loss and loss of tension associated with immobilization (disuse) in this model. Because the focus of mechanomyographic experiments was on the tibialis, only tibialis muscle weights are reported. The weight of the mice used for these experiments was 28.8 ± 1.0 g ($N = 7$). Tibialis muscle mass was significantly decreased on the immobilized compared with contralateral side (40.6 ± 2.8 vs. 50.1 ± 2.0 mg; $P < 0.01$; table 1). The significant difference between the two sides was also observed when muscle weights on both sides were normalized to body weight (1.4 ± 0.1 vs. 1.8 ± 0.1 mg/g; $P < 0.01$; respectively). Consistent with loss of muscle mass, the tension generation by the immobilized muscle was decreased compared with that by the contralateral side (34.8 ± 1.5 vs. 42.9 ± 2.0 g; $P < 0.01$). The specific tensions, tensions normalized to muscle weight and body weight, however, did not differ between the two sides (table 1).

Role of Mature AChRs in Neurotransmission during Immobilization

For the study of the role mature/immature AChRs with the use of waglerin-1, $\alpha\text{A-OIVA}$, and BTX a new set of animals were immobilized. The number of animals immobilized were 17, but only 9 were used for the studies. The animals were excluded due to death from bleeding during experiment ($n = 1$), decanulation of vein catheter ($n = 1$), inability set twitch tensions on one or other side ($n = 2$), and loosening of immobilization pin (unstable immobilization) when examined daily ($n = 4$). The mean body weight of the animals was 27.5 ± 1.0 g. The twitch tension developed by tibialis during nerve-evoked muscle contraction on the contralateral (unimmobilized) side was 36.3 ± 2.4 g, which was significantly higher ($P = 0.014$) than 29.5 ± 3.2 g on the immobilized side. Mature AChR antagonist, waglerin-1 (0.30 ± 0.05

Table 1. Tibialis Muscle Mass and Tensions at 14 Days of Immobilization

	Muscle Mass (mg)		Muscle Mass/BW* (mg/g)		Twitch Tension (g)		Specific Tension† (g/mg)		Specific Tension/BW‡ (g/mg per BW)	
	Contra	Immob	Contra	Immob	Contra	Immob	Contra	Immob	Contra	Immob
Mean	52.1	40.6	1.8	1.4	42.9	34.8	0.8	0.9	23.7	24.8
SE	2.0	2.8	0.1	0.1	1.5	1.1	0.03	0.03	1.1	0.7
P value	≤ 0.01		≤ 0.01		≤ 0.01		≤ 0.32		≤ 0.43	

Contra and Immob are contralateral and immobilized sides of the same animals.

* Muscle mass/BW is the muscle mass normalized to BW. † Specific tension is tension per gram muscle weight. ‡ Specific tension/BW is the tension per gram muscle weight normalized to BW.

BW = body weight; SE = standard error.

$\mu\text{g/g}$) caused significantly 97% or greater twitch suppression on contralateral side compared with the immobilized side ($P = 0.001$), where the tension depression was approximately 45% only (fig. 1A). The twitch tension on immobilized side could not be depressed 80% or greater with additional doses ($0.76 \pm 0.06 \mu\text{g/g}$) of waglerin-1 (or total dose of $1.06 \pm 0.12 \mu\text{g/g}$). The total dose of waglerin-1 administered was approximately $15 \times \text{ED}_{50}$ of the contralateral (normal) side ($0.07 \pm 0.06 \mu\text{g/g}$), and yet could not inhibit twitch of 80% or greater on the immobilized side. The ED doses of waglerin-1 calculated from linear regression were $0.32 \pm 0.14 \mu\text{g/g}$ versus $0.07 \pm 0.06 \mu\text{g/g}$ (ED_{50}) and 1.43 ± 0.76 versus $0.41 \pm 0.04 \mu\text{g/g}$ (ED_{95}), respectively. The ED_{95} of waglerin-1 was significantly ($P = 0.0002$) higher on the immobilized side (fig. 1B) compared with contralateral side.

Role of Immature AChRs on Neurotransmission

Previously we and others have reported that disuse by immobilization was associated *de novo* with expression of transcripts of the immature AChRs on the muscle membrane, despite the nerve being intact.^{8–12} In this component of the study, the contribution of immature AChRs to neurotransmission was assessed. In the same animals that received waglerin-1, the administration of $\alpha\text{A-OIVA}$ in a total dose of ($17.0 \pm 0.25 \mu\text{g/g}$) caused minimal changes in twitch tension on the immobilized (fig. 2) and contralateral sides. Because there was 99% twitch suppression on the contralateral side, the data for that side are not shown. Shortly thereafter, because an approximate 20% of twitch height was still present, αBTX was administered (total $0.33 \pm 0.06 \mu\text{g/g}$), and caused 97% depression of twitch or greater on the immobilized side (fig. 2).

Characterization of the Role of $\alpha 7$ AChRs in Neurotransmission

For these set of experiments, a total of eight animals were immobilized and two were not used because of ineffective immobilization. In the first set of studies ($n = 3$), after approximately 75–80% twitch depression with waglerin-1 ($0.97 \pm 0.09 \mu\text{g/g}$) and OIVA ($16.90 \pm 0.07 \mu\text{g/g}$) as described previously, methyllycaconitine was administered. Preliminary studies with methyllycaconitine indicated that the onset of effect of methyllycaconitine was relatively slow compared with that of waglerin-1. In other words, the effects of waglerin-1 on depression of tension were dissipating before onset of effect of methyllycaconitine. In view of the slower onset of methyllycaconitine, a relatively high dose was administered to produce a faster onset of effect. A total dose of $18.38 \pm 0.42 \mu\text{g/g}$ caused almost complete suppression of the remaining twitch (fig. 3). In another group of animals ($n = 3$) after stabilization of twitch tension, methyllycaconitine in a dose of $2 \mu\text{g/g}$ administered first caused 1–3% decreases in tension on the contralateral and immobilized side, respectively, in approximately 10 min (fig. 4). (The reason for this reverse order has been explained. See

Study of the role of $\alpha 7$ AChRs in Neurotransmission in Materials and Methods.) At this point, the administration of $\alpha\text{A-OIVA}$ caused minimal changes in twitch tension on either side. Waglerin ($0.20 \pm 0.00 \mu\text{g/g}$), however, caused equal twitch depression on both sides, and an additional dose of 0.08 ± 0.00 (total dose of 0.28 ± 0.00) caused complete twitch depression on both sides. In other words, once the $\alpha 7$ AChRs were preemptively blocked by methyllycaconitine, the responses to waglerin-1 were similar on the two sides.

Protein Expression of $\alpha 7$ AChRs on Muscle Membrane

The following study quantified the expression of $\alpha 7$ AChR protein on muscle membrane. Western blot analysis (fig. 5) revealed that the expression of $\alpha 7$ AChRs was increased on the immobilized compared with that in contralateral side (24.1 ± 2.9 vs. 7.9 ± 1.3 , arbitrary units, respectively; $P < 0.01$; $N = 3$ for each side). Glyceraldehyde 3-phosphate dehydrogenase, used as loading control, did not differ between the two sides. Brain extract, used as positive control, confirmed the specificity of the antibody, where the $\alpha 7$ AChRs were located at 55 kD molecular weight.

Discussion

The current study confirms the hypothesis that immobilization-induced muscle atrophy leads to *de novo* junctional-area expression of $\alpha 7$ AChRs, which play a small but significant role in neurotransmission. The significantly decreased muscle mass and/or decreased tension-generating capacity on the immobilized compared with the contralateral side (intrasubject control) in two different sets of animals confirmed the efficacy of the immobilization procedure used in these studies (table 1 and fig. 1A). With use of specific antagonists to the mature and immature AChRs, we provide evidence that a third receptor not sensitive to waglerin-1 and $\alpha\text{A-OIVA}$, but sensitive to $\alpha\text{-BTX}$ was present. The use of specific antagonist of $\alpha 7$ AChRs, methyllycaconitine, confirmed that the third receptor insensitive to waglerin-1 and $\alpha\text{A-OIVA}$ was indeed $\alpha 7$ AChRs. The immunoblot studies confirmed the expression of $\alpha 7$ AChRs in the muscle membrane. Despite the well-documented up-regulation of immature AChRs on the muscle membrane during immobilization, our studies confirm the hypothesis that the immature AChRs play a minimal role in neurotransmission on the immobilized side.

The rodent model of immobilization has been described by us previously in rats,^{9–11} and most recently in mice.²² In those studies it was demonstrated that the contralateral side behaves like naïve controls in terms of muscle function and muscle mass at 14 days of immobilization. With the use of pharmacologic probes, this study confirms that the unimmobilized contralateral side almost exclusively expresses mature receptors, evidenced by the 97% depression of tension or greater with waglerin-1 only. The contribution of

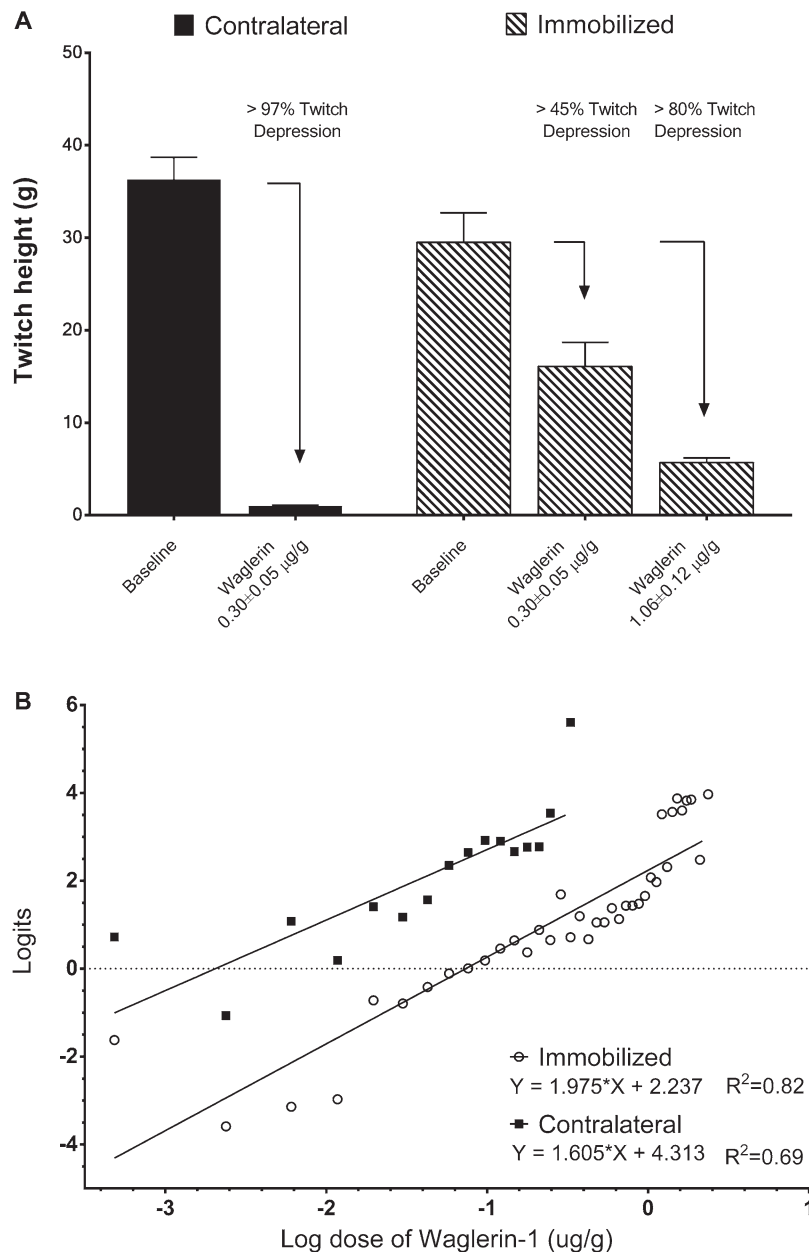


Fig. 1. (A) Immobilization leads to decreased contribution by mature acetylcholine receptors (AChRs) to neurotransmission. The baseline twitch tension on the contralateral (unimmobilized) side was 36.3 ± 2.4 g and that on the immobilized side was 29.5 ± 3.2 g, which was significantly ($P = 0.014$; $N = 9$) lower, confirming the effectiveness of immobilization. Waglerin-1 (0.30 ± 0.05 μg/g) depressed the twitch response on contralateral side to $\geq 97\%$, whereas the twitch depression on immobilized side was 45% ($P = 0.001$). Additional dose of waglerin-1 (0.76 ± 0.11 μg/g) or a total dose of 1.06 ± 0.12 μg/g could not cause twitch suppression $\geq 80\%$. This suggests that AChRs other than mature AChRs contribute to neurotransmission. (B) Dose-response curve for waglerin-1 is shifted to the right on the immobilized versus contralateral side. The cumulative log doses of waglerin-1 were plotted against percent twitch inhibition (logit plot). The doses of waglerin-1 that produced 50% depression of the first twitch of the train-of-four (T_1) effective dose (ED_{50}) on immobilized and contralateral side were 0.32 ± 0.14 μg/g and 0.07 ± 0.06 μg/g, respectively. The ED_{95} of waglerin-1 was significantly ($P = 0.0002$) higher on the immobilized side (1.43 ± 0.75 μg/g versus contralateral side (0.41 ± 0.04 μg/g) indicating resistance to the effects of waglerin-1 on the immobilized compared with the unimmobilized side.

mature AChRs to neurotransmission, however, decreases on the immobilized side, as waglerin-1 could not completely depress the twitch tension to 80% or greater. Although the up-regulation of immature receptors on the muscle

membrane has been demonstrated in many reports,^{8–12} this study demonstrates that immature AChRs contribute minimally to neurotransmission on both sides as αA -OIVA caused minimal changes in tension. In other words, the

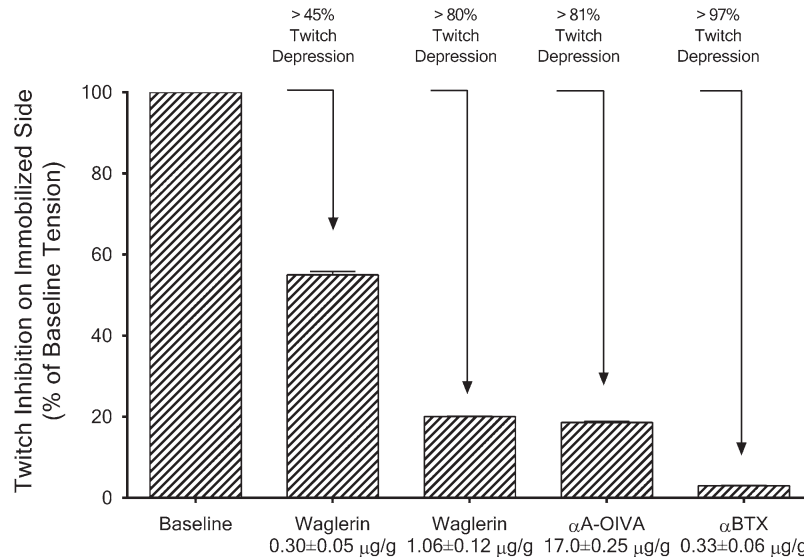


Fig. 2. Immature muscle acetylcholine receptors (AChRs) do not contribute to neurotransmission. At the end of the waglerin-1 study, the conotoxin, α A-OIVA totaling 17.0 ± 0.25 μ g/g was administered and caused minimal changes ($\leq 1\%$) in twitch tension on the immobilized side. On the contralateral side, the 99% twitch inhibition after waglerin-1 did not change after α A-OIVA (data not shown). The subsequent administration of α -bungarotoxin (α BTX) (0.33 ± 0.06 μ g/g) caused $\geq 97\%$ twitch depression on the immobilized side, indicating that an approximate 20% of the twitch height is maintained by α BTX-sensitive nicotinic AChRs. Data are presented as percent of immobilized side.

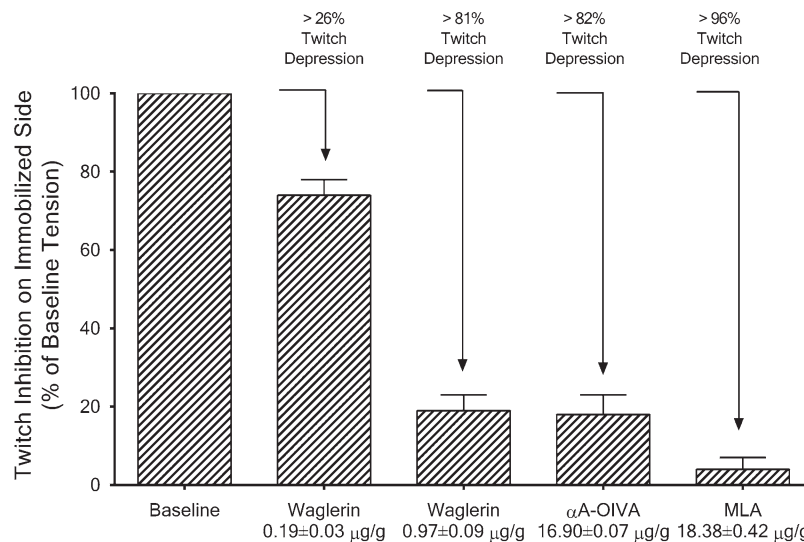


Fig. 3. The $\alpha 7$ acetylcholine receptors ($\alpha 7$ AChRs) contribute to approximately 20% twitch tension. In a separate set of animals ($N = 3$), after stabilization of twitch tension, waglerin-1 and conotoxin, α A-OIVA were administered in doses indicated in the figure until no further twitch suppression. At this point, methyllycaconitine (MLA), a specific antagonist of $\alpha 7$ AChRs, administered incrementally totaling a dose of 18.38 ± 0.42 μ g/g caused almost complete twitch suppression. Thus, approximately 20% of twitch tension during immobilization could be attributable to the nicotinic $\alpha 7$ AChRs. Data are presented as percent.

protein expression of functional immature AChRs is almost exclusively in the extrajunctional area. The fact that 20% of twitch height still remains after block with waglerin-1 and α A-OIVA suggests that this tension is maintained by receptors other than mature and immature AChRs.

Our preliminary studies with methyllycaconitine indicated that its onset of effect was slow and duration of effect prolonged relative to onset and offset of waglerin-1 effect.

Therefore, when methyllycaconitine was administered after waglerin-1 and α A-OIVA, methyllycaconitine was administered at a higher dose than when first administered. The higher dose was deliberately done to have a faster onset, before the dissipation of the effects of waglerin-1, evidenced by return of twitch height. (Higher doses of drug, particularly antagonists of muscle AChRs, can effect faster onset of paralysis.)^{3,5} In the subsequent experiment (fig. 4),

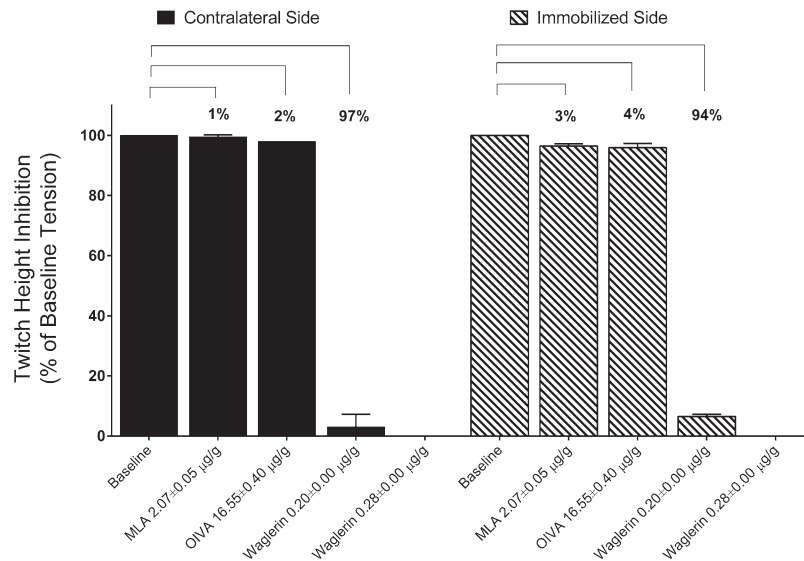


Fig. 4. Preblock of $\alpha 7$ acetylcholine receptors ($\alpha 7$ AChRs) by methyllycaconitine (MLA) causes equipotent responses to waglerin-1 on both sides. In a separate set of animals ($N = 3$), a small dose of MLA ($50 \mu\text{g}$ or $2.07 \pm 0.05 \mu\text{g/g}$) was administered first. This dose of methyllycaconitine caused minimal 1–3% twitch inhibition on both sides. After approximately 10 min, conotoxin, α A-OIVA ($16.6 \pm 0.40 \mu\text{g/g}$) caused no changes in tension on either side. The subsequent administration of waglerin-1, in doses indicated in the figure, resulted in equipotent responses on immobilized and contralateral side. Thus, a total dose of 0.28 ± 0.00 caused complete twitch suppression on both sides. Data represented as percent of baseline twitch height.

however, methyllycaconitine was administered in a smaller dose ($2 \mu\text{g/g}$ or 1/10th previous dose) at least 10 min before α A-OIVA and waglerin-1, giving sufficient time for onset of effect of methyllycaconitine. This dose caused minimal changes in twitch height. Once the $\alpha 7$ AChRs were preemptively blocked by methyllycaconitine, waglerin-1 ($0.3 \pm 0.01 \mu\text{g/g}$) was equipotent on both sides, which, therefore, reflects the pivotal role of $\alpha 7$ AChRs in the resistance to the neuromuscular effects of waglerin-1. It is notable that

α A-OIVA (fig. 4) again did not cause changes in tension, confirming the insignificant junctional expression of immature AChRs. The fact that α BTX and methyllycaconitine blocked the twitch tension after block of mature and immature AChRs indicates that inhibition of the remaining 20% twitch tension was related to the nonspecific and specific effects of α BTX and methyllycaconitine, respectively, on the $\alpha 7$ AChRs. Thus, the current study documents for the first time that $\alpha 7$ AChRs can be expressed even in innervated but pathologic muscle, and that these $\alpha 7$ AChRs receptors, but not immature AChRs, play a role in neurotransmission on the immobilized side.

One may pose the question, when administered first, why did methyllycaconitine not cause a greater twitch inhibition than observed? This question requires detailed explanation. Paton and Waud^{26,27} described the concept of margin of safety of neurotransmission in skeletal muscle. They determined that depression of twitch tension does not occur until antagonism (occupation) of 75% junctional receptors or greater. In other words, an antagonist ligand will cause twitch inhibition only when 75% or greater of the total AChRs are inhibited. Once the threshold of 75% receptor occupation is realized, twitch inhibition occurs. Because methyllycaconitine blocked only the $\alpha 7$ AChRs, there were insufficient AChRs blocked to see significant effects on twitch tension. In other words, the junctional expression of $\alpha 7$ AChRs is less than 75% of total junctional AChRs, explaining the lack of twitch inhibition, although $\alpha 7$ AChRs contribute to approximately 20% twitch tension. Western blot analyses indicated a three-fold or greater increase in protein expression of $\alpha 7$ AChRs on the immobilized muscle membrane compared

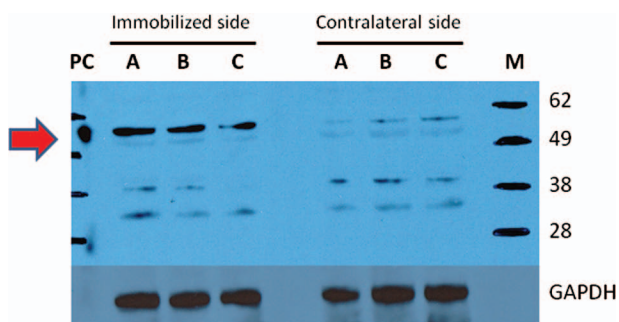


Fig. 5. Protein expression of $\alpha 7$ acetylcholine receptors ($\alpha 7$ AChRs) in muscle. The tibialis muscles from immobilized and contralateral sides ($N = 3$ per side) previously harvested were homogenized and subjected to immunoblots. Brain extract was used on positive control for $\alpha 7$ AChRs, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the loading control. The letters M and PC indicate the molecular weight marker and positive control (brain extract). The $\alpha 7$ AChRs is located at 55 kD molecular weight, indicated by the red arrow on the left. At 14 days after immobilization, the expression of $\alpha 7$ AChRs was increased three-fold compared with contralateral side.

with unimmobilized side (fig. 5). Attempts to quantitate the increased expression of immature AChRs by immunoblot proved futile due to nonspecific binding of the commercially available antibodies. No study has documented the presence of immature AChRs by using commercial antibodies. Commercially available fluorescent probes for the $\alpha 7$ AChRs also have nonspecific binding, demonstrated by the binding of these probes to brains of $\alpha 7$ AChR knockout mice.^{28,29} Thus, our study could not discriminate junctional *versus* extrajunctional expression of $\alpha 7$ AChRs, because of the nonspecific binding of the fluorescent probes. The twitch tension studies with specific antagonists, however, indicate junctional expression of $\alpha 7$ AChRs, because extrajunctional receptors play no role in neurotransmission.

The most salient finding of $\alpha 7$ AChRs expression in muscle during immobilization may have pharmacological and therapeutic implications. Resistance to the paralyzing (or neuromuscular) effects of clinically used muscle relaxants (pancuronium, atracurium) has been observed during immobilization, despite the disuse-induced muscle atrophy and muscle weakness.^{5,9,11} Although it has been postulated that the neuromuscular resistance to relaxants during immobilization is due to the expression of immature AChRs,^{3–5} the current study, by documenting the absence of functional expression of immature AChRs causing neurotransmission, debunks the theory of the role of immature AChRs in the resistance to clinically used muscle relaxants. Consistently, oocyte expression studies confirm the lack of resistance to clinically used relaxants *in vitro*.³⁰ We, therefore, posit that the resistance to clinically used muscle relaxants during immobilization is related to the expression of $\alpha 7$ AChRs. This hypothesis is consistent with the *ex vivo* observation that denervation-induced resistance to pancuronium block observed in wild-type mice was absent in $\alpha 7$ AChR knockout mice.⁷ Relative to therapeutics, stimulation of $\alpha 7$ AChRs has been shown to attenuate cytokine release and inflammation *ex vivo* and *in vivo*.^{24,31–33} Additional studies are needed to further characterize pharmacologic and therapeutic implications of $\alpha 7$ AChR expression.

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Competing Interests

The authors declare no competing interests.

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