

Mouse muscle denervation increases expression of an $\alpha 7$ nicotinic receptor with unusual pharmacology

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Neuronal nicotinic $\alpha 7$ subunits have been found in chick and rat skeletal muscle during development and denervation. In the present study, reverse transcriptase-polymerase chain reaction was used to detect $\alpha 7$ subunit mRNA in denervated mouse muscle. To determine whether the $\alpha 7$ subunit forms functional nicotinic acetylcholine receptors (nAChRs) in muscle, choline was used to induce a membrane depolarization because choline has been considered a specific agonist of $\alpha 7$ -containing ($\alpha 7^*$) nAChRs. We found, however, that choline (3–10 mM) also weakly activates muscle nAChRs. After inhibiting muscle nAChRs with a specific muscle nAChR inhibitor, α -conotoxin GI (α CTxGI), choline was used to activate the $\alpha 7^*$ nAChRs on muscle selectively. Four weeks after denervation, rapid application of choline (10 mM) elicited a substantial depolarization in the presence of α CTxGI (0.1 μ M). This component of the depolarization was never present in denervated muscles obtained from mutant mice lacking the $\alpha 7$ subunit (i.e. $\alpha 7$ -null mice). The depolarization component that is resistant to α CTxGI was antagonized by pancuronium (3–10 μ M) and by a 4-oxystilbene derivative (F3, 0.1–0.5 μ M) at concentrations considered highly specific for $\alpha 7^*$ nAChRs. Another selective $\alpha 7$ antagonist, methyllycaconitine (0.05–5 μ M), did not strongly inhibit this choline-induced depolarization. Furthermore, the choline-sensitive nAChRs showed little desensitization over 10 s of application with choline (10–30 mM). These results indicate that functional $\alpha 7^*$ nAChRs are significantly present on denervated muscle, and that these receptors display unusual functional and pharmacological characteristics.

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Nicotinic acetylcholine receptors (nAChRs) are pentameric membrane proteins that form cation-selective ion channels (McGehee & Role, 1995; Lindstrom *et al.* 1996; Role & Berg, 1996; Albuquerque *et al.* 1997; Dani, 2001). There are two broad classes of nAChRs based on their general location: muscle nAChRs composed of $\alpha 1$, $\beta 1$, δ , and γ or ϵ subunits, and neuronal nAChRs formed by $\alpha 2$ – $\alpha 10$ and $\beta 2$ – $\beta 4$ subunits (Cordero-Erausquin *et al.* 2000). This classification is not definitive, however, because neuronal nAChRs have been found in non-neuronal cells (Grando *et al.* 1995; Navaneetham *et al.* 1997; Flora *et al.* 2000), and the $\alpha 1$ muscle nAChR subunit has been found in chick ciliary ganglia (Pugh *et al.* 1995).

The expression of nAChRs in skeletal muscle is regulated by neural activity during development (Hall & Sanes, 1993). In the embryonic state, the most abundantly expressed nAChR is a muscle-type receptor composed of $\alpha 1$, $\beta 1$, γ , δ . Small quantities of neuronal nAChR subunits have also been found in chick ($\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 4$) and rat ($\alpha 7$) muscle (Corriveau *et al.* 1995; Romano *et al.* 1997; Fischer *et al.* 1999). As the neuromuscular junction develops, the ϵ subunit replaces the γ subunit in the muscle nAChR, and neuronal nAChR subunits are no longer expressed. Upon

denervation, the γ subunit replaces the ϵ subunit in the muscle nAChR, and the neuronal $\alpha 7$ subunit is expressed again (Romano *et al.* 1997; Fischer *et al.* 1999).

The $\alpha 7$ subunit can form a homomeric nAChR channel when expressed in *Xenopus* oocytes (Séguéla *et al.* 1993). $\alpha 7$ homomeric channels are highly permeable to Ca^{2+} and are inhibited by α -bungarotoxin (α -BgTx) (Séguéla *et al.* 1993; Zhang *et al.* 1994; Castro & Albuquerque, 1995). Several biochemical and biophysical studies have demonstrated that the native α -BgTx-binding sites are composed solely of $\alpha 7$ subunits in rat neuronal cells (Chen & Patrick, 1997; Rangwala *et al.* 1997). However, the $\alpha 7$ subunit can participate in heteromeric nAChRs in chick retinal ganglion neurons ($\alpha 7\alpha 8^*$ nAChR; Anand *et al.* 1993) and in chick sympathetic neurons ($\alpha 3\alpha 7^*$ and $\alpha 3\alpha 5\alpha 7^*$ nAChRs; Yu & Role, 1998b). In *Xenopus* oocytes, heterologously expressed $\alpha 7$ subunits can also contribute to the formation of heteromeric nAChRs, such as $\alpha 7\beta 1\gamma\delta$ (Helekar *et al.* 1994) and $\alpha 7\beta 3$ (Palma *et al.* 1999). Thus, the native $\alpha 7^*$ nAChRs are not necessarily homogeneous in the nervous system. There might be cell-specific regulatory mechanisms that control the composition and function of $\alpha 7^*$ nAChRs (see Cooper & Millar, 1997).

Recently, it was proposed that the $\alpha 7$ subunit might form a functional nAChR in the denervated muscle of the rat (Fischer *et al.* 1999), but its function was not fully identified. In the present study, we confirm the expression of the $\alpha 7$ subunit in denervated mouse muscle by reverse transcriptase-polymerase chain reaction (RT-PCR). Using mutant mice that lack the $\alpha 7$ subunit ($\alpha 7$ -null mice), we provide evidence indicating that the $\alpha 7$ subunit contributes to a nAChR in muscle that has an unusual pharmacology.

METHODS

Animals

C57BL/6J mice were bred and kept in the animal-care facilities of our institution. Animals were housed (3–5 per cage) under a 12 h–12 h light–dark cycle with free access to water and food. In some electrophysiological recordings (Figs 2, 4 and 6), we used mice lacking the $\alpha 7$ subunit. These $\alpha 7$ -null mice were generated by mating heterozygous parents. The offspring were genotyped by PCR (Orr-Urtreger *et al.* 1997) using the following primers: $\alpha 7(+)$, CCT GGT CCT GCT GTG TTA AAC TGC TTC; $\alpha 7(-)$, CTG CTG GGA AAT CCT AGG CAC ACT TGA G; and Neo-3(-), CTG ATC GAC AAG ACC GGC TTC CAT CC. PCR was performed as follows: 94 °C, 5 min; 30 cycles of 94 °C, 1 min; 68 °C, 1 min; 72 °C, 1.5 min; then 72 °C, 5 min. The $\alpha 7$ -null (-/-) mutant mice were compared to their $\alpha 7$ (+/+) wild-type siblings. After PCR, 30 μ l of the PCR products were run on a 2% agarose gel. The wild-type band (primers $\alpha 7(+)$ and $\alpha 7(-)$) was 435 bp, the mutant band (primers $\alpha 7(+)$ and Neo-3(-)) was 750 bp.

All procedures were approved by the Baylor College of Medicine Animal Research Committee and followed the 'Using Animals in Intramural Research' guidelines from the National Institutes of Health.

Muscle denervation

Mice (2–3 months old) were anaesthetized with a mixture of ketamine (112.5 mg kg⁻¹), xylazine (5.7 mg kg⁻¹) and acepromazine (1.1 mg kg⁻¹) injected intraperitoneally. The hind limbs were surgically denervated by removing approximately 1 cm of the sciatic nerve just proximal to the bifurcation of the tibial and peroneal nerve. Four weeks after the denervation, soleus muscles were isolated.

RT-PCR

Total cellular RNA was extracted from mouse (2–4 months old) tissue using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The following sets of primers were used, as described previously (Léna *et al.* 1999; Song *et al.* 1999): $\alpha 7$ forward: 5'-GTG GAA CAT GTC TGA GTA CCC CGG AGT GAA-3', $\alpha 7$ reverse: 5'-GAG TCT GCA GGC AGC AAG AAT ACC AGC A-3', β -actin forward: 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3', β -actin reverse: 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'. These sets of primers were located on different exons to rule out genomic DNA amplification. RT-PCR for detection of $\alpha 7$ subunit and β -actin mRNA was performed in individual tubes using 0.3 μ g total RNA and the SuperScript One-Step RT-PCR kit (Life Technologies, Grand Island, NY, USA) with the following conditions. The reverse transcriptase reaction was performed at 50 °C for 30 min. After an initial denaturation step at 94 °C for 2 min, 37 and 33 cycles (94 °C, 30 s; 60 °C, 45 s; 68 °C, 45 s) of PCR were run to obtain the $\alpha 7$ and β -actin products, respectively. The numbers of PCR cycles chosen were within the linear range of the

amplification. The number of PCR cycles was chosen to obtain a band strong enough for easy quantification, but still faint enough to observe a linear increase in PCR product in the gel when more cycles were used. The number of PCR cycles that were tested for linearity was 35–41 and 33–40, respectively, before settling on the values given. Subsequently, 20 μ l of each PCR product was run on 1% agarose gels containing ethidium bromide. Fluorescence intensity from the PCR bands on the gels was measured using an imaging system (Alpha Innotech, San Leandro, CA, USA) with ChemiImager version 5.5 software. Data are expressed as means \pm S.E.M. Statistical differences were determined by Student's unpaired *t* test (two tails).

Electrophysiology

Adult mice (2–4 months old) were anaesthetized with halothane and killed by decapitation. Diaphragm and soleus muscles were isolated with their tendons. The muscle preparation was stretched slightly beyond its resting tension, and was fixed with insect pins on rubber plates in a chamber. A modified Krebs solution (mM: NaCl 122, KCl 5.9, CaCl₂ 2.5, MgCl₂ 1.2, NaHCO₃ 15.5, and glucose 11.5, pH 7.4) was equilibrated with 95% O₂ and 5% CO₂, maintained at 32 \pm 1 °C and perfused through the chamber (2 ml min⁻¹). Intracellular recordings were performed in current-clamp mode using an Axopatch-1C (Axon Instruments, Foster City, CA, USA) with a glass micropipette filled with 3 M KCl (20 M Ω resistance). The microelectrode was inserted near the endplate on the surface fibres such that the rise times of the miniature endplate potentials were 1 ms or less. To minimize the differences among the preparations used, the membrane potential was corrected by passing currents into the muscle fibre through the recording pipettes. Experiments were conducted at a membrane potential of approximately -60 mV in innervated muscle. In the case of denervated muscle, the entire surface of the muscle exhibits supersensitivity to acetylcholine (ACh) (Cangiano, 1985). We recorded from regions mid-way along the muscle fibre, as described by Fischer *et al.* (1999). The resting membrane potential was lowered in denervated muscle, such that the experiments were conducted at a membrane potential of approximately -53 mV.

In order to measure the depolarization evoked by nicotinic agonists, such as choline and ACh, local agonist applications were made onto the muscle membrane 0.2 mm away from the tip of the recording electrode. The agonist was rapidly applied with outflow tubes (150 μ m i.d. glass pipettes) mounted on a computer-controlled, high-speed motorized drive (Newport Corp., Irvine, CA, USA) (see Vernino *et al.* 1992, 1994). The duration of the local agonist application was 30 ms, unless otherwise mentioned. The external solution contained 1 μ M atropine, a muscarinic ACh receptor antagonist, throughout the measurement to prevent any involvement of muscarinic receptors in the observed responses. To block muscle-type nAChRs, the preparations were often pretreated with α -conotoxin GI (α CTxGI) by bath application for at least 20 min before application of the nicotinic agonists. For the experiments investigating the pharmacological properties of the agonist-induced depolarization that was resistant to α CTxGI, pancuronium (20 min), methyllycaconitine (MLA, 20 min), F3 (30 min) or α -BgTx (30 min) was bath applied in combination with α CTxGI before recording. These antagonists were continuously present in the chamber during and after the local application of pure agonist solution (30 ms duration). We also performed experiments with the antagonist in both the bath and the outflow tube to rule out the possibility that the blocking effect of a nicotinic antagonist would be weakened as the pure choline

solution was applied via the outflow tube. We investigated the choline response with MLA (50 nM to 1 μ M) and α CTxGI (0.1 μ M) added not only to the chamber but also to the choline solution (10–30 mM) in the outflow tube (Table 1). While testing for desensitization using long (1–10 s) choline applications, 0.1 μ M α CTxGI was present both in the bath and in the outflow solution.

Data acquisition and analysis (Axobasic, Axon Instruments) were performed using a personal computer equipped with an interface board (TL-1, Axon Instruments). Electrophysiological responses were sampled at a rate of 67 Hz for 4096 sample points. To assess the effects of drugs, peak amplitudes were compared. We estimated the peak amplitude of an agonist-induced depolarization (mV) by calculating the difference between the resting membrane potential and the peak of the depolarization. The resting potential was obtained by averaging the baseline for 4 s just before applying the nicotinic agonist. Within each muscle, measurements were considered independent and contributed to the *n* value when new muscle fibres were used. The data were then averaged and expressed as means \pm S.E.M. The number of muscle fibres tested was given as *n*, and the number of different muscles that were used is also given. Statistical differences were determined by Student's unpaired *t* test (two tails), unless otherwise mentioned.

Reagents

All chemicals were purchased from Sigma (St Louis, MO, USA), unless indicated. MLA was purchased from Research Biochemicals International (Natick, MA, USA). α -CTxGI was purchased from Calbiochem-Novabiochem (La Jolla, CA, USA). F3 was kindly provided by Dr C. Gotti (University of Milan, Italy).

RESULTS

Expression of nAChR $\alpha 7$ subunit mRNA in denervated muscle

RT-PCR was performed to assess mRNA expression of β -actin and nAChR $\alpha 7$ subunit (Fig. 1). Since the expression level of β -actin in skeletal muscle is not modified after denervation (Anzai *et al.* 1996), the amount of $\alpha 7$ PCR product was normalized as a percentage of the β -actin level. A fragment of neuronal $\alpha 7$ subunit of the expected size was obtained in 4 out of 14 normal (innervated) soleus muscles (29%) of adult mice. The expression level of $\alpha 7$ mRNA was not changed 2 weeks after denervation (data not shown). Four weeks after muscle denervation, the $\alpha 7$ PCR product was found in a higher proportion (7 out of 9 denervated muscles, 78%), and the mean amplified levels were significantly increased from those in the innervated muscle (Fig. 1).

Although there is a report indicating that $\alpha 7$ mRNA is expressed in human mononuclear leukocytes (Kawashima & Fujii, 2000), no $\alpha 7$ subunit was detected from blood samples (200 μ l per mouse, *n* = 10), which we used as a negative control. This result also indicates that $\alpha 7$ PCR products detected in muscle preparations were not derived from the blood cells contained in microvessels in muscle. On the other hand, a large amount of $\alpha 7$ PCR product was obtained in all hippocampal tissues isolated from mouse brain (*n* = 6). The hippocampus serves as a positive control

because $\alpha 7^*$ nAChR is highly expressed there (Albuquerque *et al.* 1997; Jones *et al.* 1999; Dani, 2001; Ji *et al.* 2001). No differences were detected in the size of fragments amplified from muscle and hippocampal preparations. These negative and positive controls support the specificity of our detection of $\alpha 7$ mRNA in muscle.

Choline-evoked depolarizations mediated by muscle-type nAChRs in innervated muscle

Intracellular recordings were performed to investigate the possibility that the $\alpha 7$ subunit forms a functional nAChR in denervated skeletal muscle. All the nicotinic responses analysed in this study returned to the resting membrane potential within 3 min after stimulation, and were stable and repeatable.

Choline is a full agonist for $\alpha 7^*$ nAChRs, and is considered a very weak agonist for muscle-type nAChRs (Papke *et al.* 1996; Alkondon *et al.* 1997). However, the application of 10 mM choline elicited a depolarization at neuromuscular

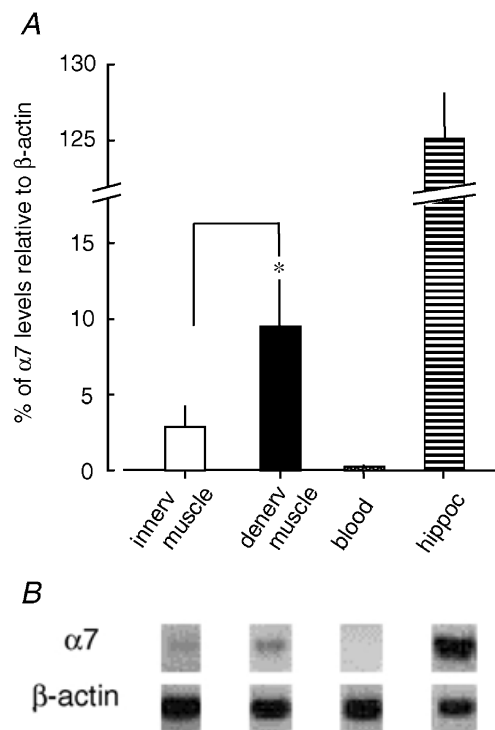


Figure 1. Semi-quantified expression level of the $\alpha 7$ nAChR subunit by RT-PCR

Total RNA was extracted from mouse innervated muscle (innerv), 4 week-denervated muscle (denerv), blood and hippocampus (hippoc). RT-PCR products for $\alpha 7$ subunit and β -actin were obtained simultaneously from each sample. A, the $\alpha 7$ mRNA level was normalized as a percentage of the β -actin mRNA level, which was considered to be 100%. Data values represent the means \pm S.E.M. (*n* = 6–14). A significant difference ($* P < 0.05$) existed between innervated and denervated muscles as determined by an unpaired *t* test. B, photo-images in the negative form demonstrating the RT-PCR product for $\alpha 7$ mRNA that yielded the expected band of 509 bp, and the product for β -actin mRNA that showed the expected band of 348 bp.

endplates in innervated diaphragm muscle of mice (Fig. 2A). The choline-evoked response developed slowly (time to peak, 5.0 ± 0.8 s, $n = 18$, 7 muscles) and recovered slowly (1/3 decay time or time to decline to 66.7% of peak amplitude, 13.9 ± 2.3 s, $n = 18$, 7 muscles). The response was faster when 0.3 mM ACh was applied to the endplate: time to peak, 1.1 ± 0.2 s, $n = 12$, 3 muscles, $P < 0.001$; 1/3 decay time, 2.5 ± 0.2 s, $n = 12$, 3 muscles, $P < 0.001$ (Fig. 2B). It was necessary to use > 15-times higher concentrations of choline than ACh to obtain a similar amplitude of depolarization (Fig. 2C and D). Moreover, the peak amplitude of the choline-evoked depolarization from innervated muscles of $\alpha 7^{+/+}$ mice was the same as that in $\alpha 7^{-/-}$ mice (Fig. 2C). These results indicate that the agonist activity of choline on muscle-type nAChRs is independent of $\alpha 7^*$ nAChRs.

To verify that muscle-type nAChRs underlie the endplate depolarization by choline, innervated diaphragm muscles were pretreated with α CTxGI, which specifically inhibits muscle-type nAChRs expressed in *Xenopus* oocytes

($IC_{50} = 20$ nM) without affecting $\alpha 7$ homomeric nAChRs (Johnson *et al.* 1995). At the concentrations (10–100 nM) used previously (Johnson *et al.* 1995), α CTxGI inhibited both choline-evoked and ACh-evoked depolarizations (Fig. 2E and F). This result supports the conclusion that choline can activate muscle-type nAChRs to cause a depolarization of innervated muscle at neuromuscular endplates.

Nicotinic depolarizations in denervated muscle that are independent of muscle-type nAChRs

To investigate the function of $\alpha 7^*$ nAChRs in adult mouse muscle, α CTxGI was used to inhibit muscle-type nAChRs. α CTxGI (0.1 μ M) almost completely inhibited choline-evoked depolarizations at the endplates of innervated mouse soleus muscle (Fig. 3A, upper trace). However, 4 weeks after muscle denervation, the application of 10 mM choline evoked a significant depolarization all along the surface of the muscle membrane even in the presence of 0.1 μ M α CTxGI (Fig. 3A, lower trace). Most of the denervated muscle responded to the choline

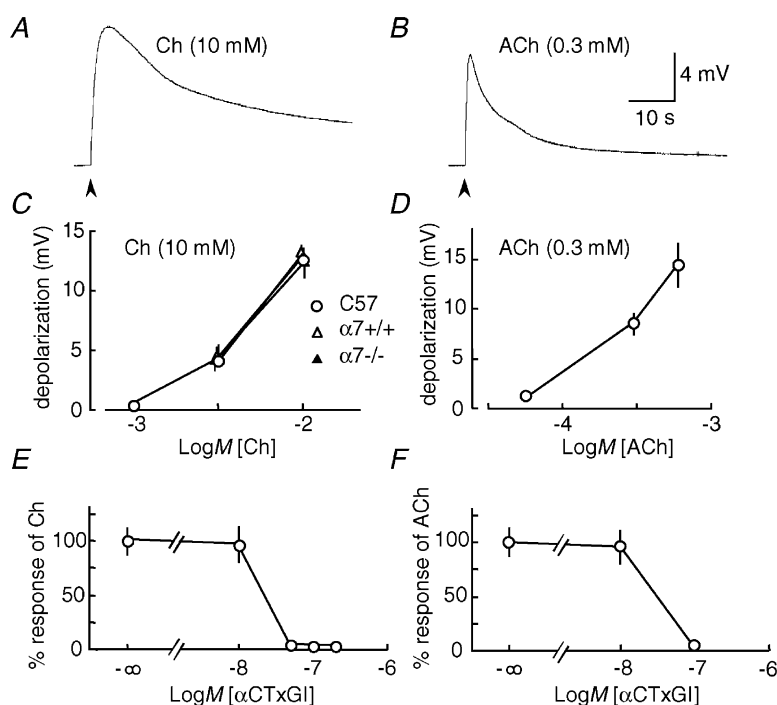


Figure 2. Choline and ACh evoke depolarizations mediated by muscle-type nAChRs at the endplate of innervated muscle

Rapid agonist applications were made to fibres from 3 mouse diaphragm muscles. *A*, trace showing the averaged response to 10 mM choline (Ch; $n = 16$). The arrowhead indicates agonist application. *B*, averaged response to 0.3 mM ACh ($n = 12$). *C*, concentration–response curves for choline from C57BL/6J mice ($n = 4–16$), $\alpha 7^{+/+}$ ($n = 13–21$) and $\alpha 7^{-/-}$ mice ($n = 9–21$). *D*, concentration–response curve of ACh from C57BL/6J mice ($n = 5–12$). The peak amplitude of the depolarizations evoked by agonists was calculated and then averaged. *E*, concentration–response curve demonstrating inhibition by α -conotoxin GI (α CTxGI) of the depolarization evoked by 10 mM choline ($n = 3–8$). *F*, concentration–response curve demonstrating inhibition by α CTxGI of the depolarization evoked by 0.3 mM ACh ($n = 4–12$). These responses were obtained 20 min after treatment with α CTxGI. The peak amplitude of depolarization was normalized as a percentage of the control response observed without α CTxGI. Data values in *C–F* represent the means \pm S.E.M.

application (16 of 17 recordings, 5 muscles), and the averaged peak amplitude of depolarization was significantly increased when compared to the response from the innervated muscles (Fig. 3B). In addition, choline increased the peak amplitude of the α CTxGI-resistant depolarization in a concentration-dependent manner: the peak amplitude was 3.2-fold augmented by increasing the choline concentration from 10 to 30 mM (Table 1). Using a higher concentration of α CTxGI (1 μ M) did not inhibit this component of the choline-evoked depolarization (Fig. 3B), indicating that muscle-type nAChRs do not mediate this component.

ACh also evoked a depolarization in the denervated soleus muscles in the presence of 0.1 μ M α CTxGI (Fig. 3C). The peak amplitude of the depolarization was augmented by increasing ACh from 0.06 to 0.6 mM (not shown). On the other hand, the depolarization caused by ACh (0.3 mM) was not altered by increasing α CTxGI from 0.1 to 0.5 μ M (Fig. 3D). The α CTxGI-resistant depolarization evoked by 0.3 mM ACh was 5-times larger than that evoked by 10 mM choline in denervated muscle (Fig. 3B and D). The ACh–choline relationship was different at the innervated muscle

Table 1. No effect of methyllycaconitine on the α CTxGI-resistant depolarization by choline in denervated muscle

Choline (mM)	MLA (μ M)	Depolarization (mV)	<i>n</i>	Muscles tested
10	0	0.72 \pm 0.23	11	5
10	1	0.75 \pm 0.26 n.s.	11	4
30	0	2.31 \pm 0.20	8	3
30	0.05	2.31 \pm 0.28 n.s.	9	3

The denervated muscles were pretreated with methyllycaconitine (MLA) in the presence of α CTxGI (0.1 μ M). Then, the choline solution (10–30 mM) containing MLA (in the same concentration as the bath solution, i.e. 0.05 or 1 μ M) and α CTxGI (0.1 μ M) was rapidly applied via the outflow tube. n.s., not significantly different from the control response without MLA, determined by unpaired *t* test.

endplate where 0.3 mM ACh and 10 mM choline evoked similarly sized depolarizations from muscle-type junctional nAChRs (Fig. 2B and D).

The α CTxGI-resistant component differed from the muscle-type response in other ways. The time to peak and

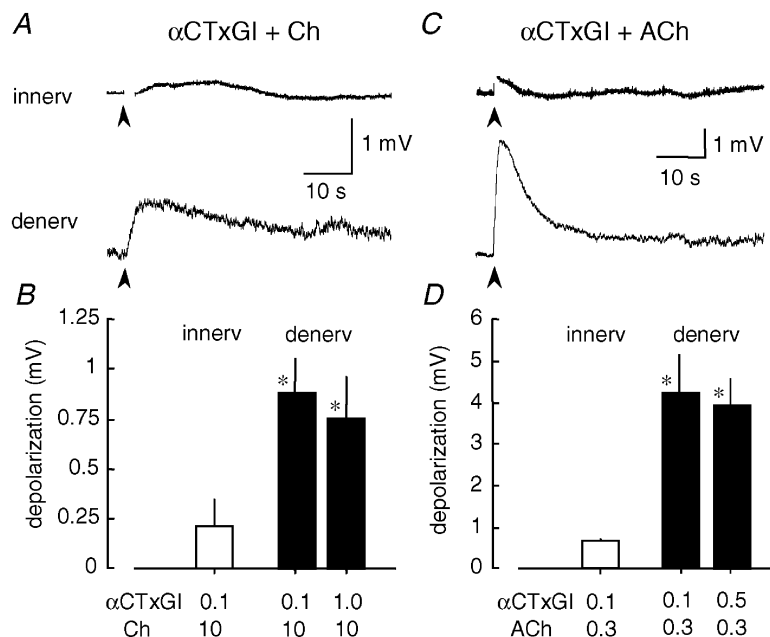


Figure 3. The α CTxGI-resistant depolarization by choline and ACh in denervated muscle of C57BL/6J mice

All the data were obtained after treatment with α CTxGI in mouse soleus muscles. *A*, traces showing averaged response to 10 mM choline (Ch) in the presence of 0.1 μ M α CTxGI in the innervated (innerv) and denervated (denerv) muscles ($n = 10$ –17, 4–5 muscles). Stimulation artifacts just after the agonist applications (arrowheads) are blanked. *B*, peak amplitude of 10 mM choline-evoked depolarization in the presence of α CTxGI (0.1–1 μ M) in the innervated and denervated states. Data values represent the means \pm S.E.M. ($n = 10$ –17). *C*, traces demonstrating averaged response to 0.3 mM ACh in the presence of 0.1 μ M α CTxGI in the innervated and denervated muscles. Stimulation artifacts are blanked ($n = 3$ –6, 3 muscles). *D*, peak amplitude of 0.3 mM ACh-evoked depolarization in the presence of α CTxGI (0.1–0.5 μ M) in the innervated and denervated states. Data values represent the means \pm S.E.M. ($n = 3$ –6). *Significantly different response ($P < 0.05$) to choline or ACh in the presence of 0.1 μ M α CTxGI in the denervated muscles from those in the innervated muscle, determined by unpaired *t* test.

1/3 decay time of the α CTxGI-resistant component were 9.5 ± 1.4 and 15.4 ± 3.7 s, respectively ($n = 16$, 5 muscles) when evoked by choline (10 mM) and were 2.0 ± 0.4 s ($n = 6$, 3 muscles, $P < 0.01$) and 3.5 ± 0.4 s ($n = 6$, 3 muscles, $P < 0.05$ determined by Student's unpaired one-tail t test) when evoked by ACh (0.3 mM). These times are significantly longer than those found with junctional nAChRs. For example, when compared with the innervated endplate response, the time to peak of the α CTxGI-resistant component in denervated muscle was 1.9-fold longer in 10 mM choline and 1.8-fold longer in 0.3 mM ACh. These results support the conclusion that, in denervated muscle treated with α CTxGI, the choline-evoked or ACh-evoked membrane depolarizations are not mediated by muscle-type nAChRs.

$\alpha 7$ -Null mice lack the α -CTxGI-resistant depolarization evoked by choline in denervated muscle

We used $\alpha 7$ -null ($-/-$) mutant mice to identify the nAChR subtype involved in the choline-evoked, α CTxGI-resistant depolarization. In wild-type littermates ($\alpha 7+/+$ mice), 10 mM choline (with $0.1 \mu\text{M}$ α CTxGI) elicited a depolarization in denervated soleus muscles ($n = 17$,

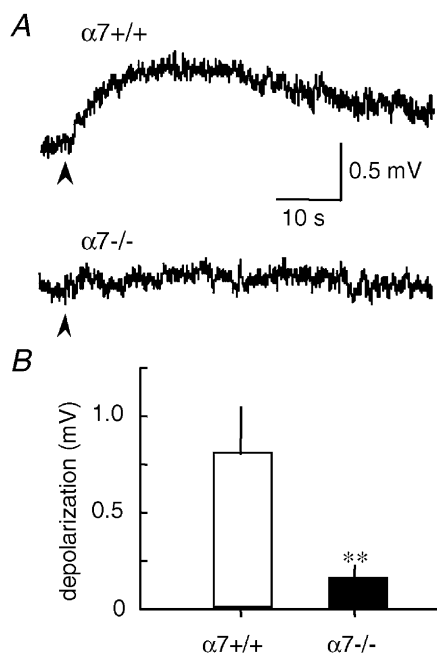


Figure 4. Lack of the α CTxGI-resistant response to choline in denervated muscle from $\alpha 7$ -null mice

Choline-evoked depolarization was obtained after treatment with $0.1 \mu\text{M}$ α CTxGI in the denervated soleus muscles. *A*, traces showing averaged response to 10 mM choline in $\alpha 7+/+$ and $\alpha 7-/-$ mice. Stimulation artifacts caused by the agonist applications (arrowheads) have been eliminated. *B*, peak amplitude of the α CTxGI-resistant depolarization by choline in $\alpha 7+/+$ and $\alpha 7-/-$ mice. Data values represent the means \pm S.E.M. ($n = 17-24$, 5-6 muscles, $**P < 0.01$, determined by unpaired t test).

5 muscles; Fig. 4). This component of depolarization was statistically the same in amplitude as the response found with C57BL/6J wild-type mice (Fig. 3). In contrast, no significant α CTxGI-resistant depolarization in response to choline was ever observed with denervated soleus muscles from $\alpha 7$ -null ($-/-$) mice ($n = 24$, 6 muscles; Fig. 4*A* and *B*). These results strongly support the idea that this α CTxGI-resistant depolarization in denervated muscle requires the $\alpha 7$ subunit and $\alpha 7^*$ nAChRs.

Pharmacological properties of the α -CTxGI-resistant depolarization

The pharmacological properties of the choline-evoked depolarizations in innervated diaphragm muscle (no α CTxGI) and denervated muscle (with α CTxGI) were compared. When pretreated with pancuronium (0.1–10 μM),

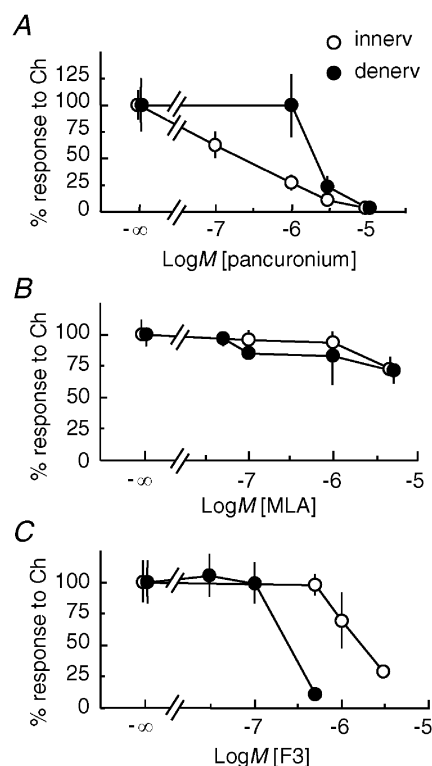


Figure 5. Distinct pharmacological properties of the α CTxGI-resistant depolarization in denervated muscle and the junctional nicotinic response in innervated muscle

Choline-evoked depolarizations were obtained in either the innervated diaphragm muscles without α CTxGI (innerv, \circ) or the denervated soleus muscles treated with $0.1 \mu\text{M}$ α CTxGI (denerv, \bullet) in C57BL/6J mice. The inhibitors (pancuronium, MLA or F3) were applied first, before rapid agonist application was carried out. The concentration–response curves demonstrate the inhibitory effects of pancuronium (*A*), MLA (*B*) and F3 (*C*) on the depolarization induced by 10 mM choline (Ch). Peak amplitude of the depolarization was normalized as a percentage of the control response obtained without these blockers. Data values represent the means \pm S.E.M. (*A*: \circ , $n = 4-8$, 3 muscles; \bullet , $n = 7-9$, 4 muscles; *B*: \circ , $n = 17-18$, 3 muscles; \bullet , $n = 3-4$, 3 muscles; *C*: \circ , $n = 3-5$, 3 muscles; \bullet , $n = 6-8$, 3 muscles).

a competitive nAChR antagonist, the peak amplitudes of both responses were diminished in a concentration-dependent manner (Fig. 5A), but the effective concentration ranges differed. The IC_{50} for the $\alpha CTxGI$ -resistant component in the denervated muscle was $2.1 \mu M$, whereas the IC_{50} for the depolarization in the innervated muscle was $0.2 \mu M$. The IC_{50} in denervated muscle was similar to the IC_{50} ($3 \mu M$) obtained by whole-cell patch-clamp recordings of ACh-induced $\alpha 7^*$ currents from rat hippocampal interneurons (not shown), whereas the IC_{50} in the innervated muscle was similar to that of junctional muscle-type ($\alpha 1\beta 1\epsilon\delta$) nAChRs expressed in *Xenopus* oocytes (Yost & Winegar, 1997).

In hippocampal neurons, MLA at 1–5 nM effectively inhibits $\alpha 7$ nAChRs (Alkondon *et al.* 1992; Palma *et al.* 1996; Ji *et al.* 2001). MLA at much higher concentrations (0.05–5 μM) did not strongly inhibit either the $\alpha CTxGI$ -resistant depolarization in the denervated muscle or the junctional response in the innervated muscle (Fig. 5B). MLA (5 μM) diminished the peak amplitude of both responses by only 30%. In these experiments, choline was applied to the muscle fibre via an outflow tube, and the MLA was present in the chamber but not the outflow tube. To exclude the possibility that the MLA was being washed off as the choline solution arrived from the outflow tube, MLA (1 μM) and $\alpha CTxGI$ (0.1 μM) were added not only to the chamber but also to the choline solution (10–30 mM) in the outflow tube. The results were the same: the peak amplitude of the $\alpha CTxGI$ -resistant response with MLA was still not different from the control response without MLA (Table 1).

This surprisingly weak effect of MLA on the $\alpha CTxGI$ -resistant component led us to use another potent antagonist for $\alpha 7^*$ nAChRs, the 4-oxystilbene derivative F3 (Gotti *et al.* 1998, 2000). Pretreatment with F3 decreased the peak amplitude of the $\alpha CTxGI$ -resistant component. Both responses to choline were inhibited but with different concentration dependencies (Fig. 5C). As expected for an $\alpha 7^*$ nAChR (Gotti *et al.* 1998, 2000), the F3 concentrations used to inhibit the $\alpha CTxGI$ -resistant component (0.1–0.5 μM , $IC_{50} = 0.26 \mu M$) were 6-times lower than those used to inhibit the junctional nAChRs of innervated muscle (1–3 μM , $IC_{50} = 1.6 \mu M$).

To further characterize the difference between the junctional nAChRs and those mediating $\alpha CTxGI$ -resistant depolarization, we used α -BgTx, an antagonist of both $\alpha 7^*$ and muscle-type nAChRs. The peak amplitude of the $\alpha CTxGI$ -resistant depolarization evoked by 10 mM choline was significantly diminished by 0.1 μM α -BgTx. The control response without α -BgTx was 1.02 ± 0.19 mV ($n = 19$, 7 muscles), and the test response with α -BgTx was 0.25 ± 0.09 mV ($n = 13$, 5 muscles, $P < 0.01$ determined by unpaired *t* test). The junctional nAChR responses evoked by 10 mM choline in the innervated muscle were

completely blocked by 0.1 μM α -BgTx ($n = 6$, 3 muscles). Although both the junctional nAChRs and the putative $\alpha 7^*$ nAChR are inhibited by α -BgTx, the concentration dependence is slightly different. Thus, consistent with the functional data, the pharmacology supports the conclusion that the $\alpha CTxGI$ -resistant depolarization in denervated muscle is not mediated by typical muscle-type nAChRs.

Desensitization profile of the α -CTxGI-resistant depolarization

$\alpha 7^*$ nAChRs desensitize rapidly (< 100 ms) upon prolonged application of a high concentration of agonist (Alkondon *et al.* 1997). We therefore examined the effects of prolonged exposure to choline on the $\alpha CTxGI$ -resistant depolarization in denervated muscle. When the exposure time was increased to 1 s, 10 mM choline induced a sustained depolarization in denervated muscles of $\alpha 7^{+/+}$ mice in the presence of

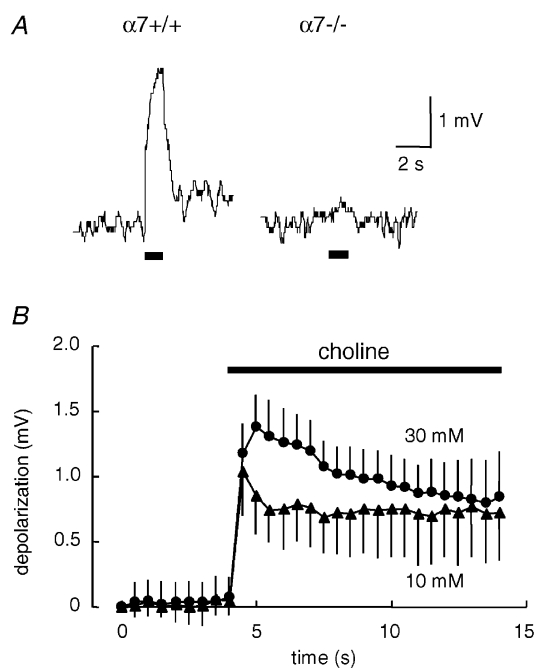


Figure 6. $\alpha CTxGI$ -resistant depolarization present throughout prolonged application of choline in denervated muscle

Choline-evoked depolarization was obtained after treatment with 0.1 μM $\alpha CTxGI$ in the denervated soleus muscles. *A*, traces showing typical recordings of the $\alpha CTxGI$ -resistant depolarization evoked by 1 s application of choline (10 mM) in denervated muscles of $\alpha 7^{+/+}$ and $\alpha 7^{-/-}$ mice. Horizontal bars below the traces indicate 1 s application of choline. Similar results were obtained in 3 separate muscle fibres from 2 muscles of $\alpha 7^{+/+}$ mice and in 5 separate muscle fibres from 2 muscles of $\alpha 7^{-/-}$ mice. *B*, time course of averaged $\alpha CTxGI$ -resistant responses to prolonged choline application in denervated muscles of $\alpha 7^{+/+}$ mice. The choline solution (10–30 mM) containing 0.1 μM $\alpha CTxGI$ was continuously applied to the recording area for 10 s via an outflow tube. The horizontal bar in the plot indicates 10 s application of choline. Data values represent the means \pm S.E.M. (10 mM choline: \blacktriangle , $n = 11$, 4 muscles; 30 mM choline: \bullet , $n = 10$, 6 muscles).

0.1 μM αCTxGI , whereas the choline response was nearly absent in $\alpha 7^{-/-}$ mice (Fig. 6A). When the exposure time was further increased to 10 s, the αCTxGI -resistant depolarization declined with time in denervated muscles of C57BL/6J mice (Fig. 6B): during the 10 s of application, the response to 10 mM choline decreased by 31 % from the peak amplitude, and the response to 30 mM choline decreased by 39 % from the peak amplitude. In other words, the depolarization only partially desensitized, and the majority of the current persisted throughout the 10 s choline application. Thus, unlike classical neuronal $\alpha 7^*$ nAChR responses, the αCTxGI -resistant depolarization did not exhibit a fast desensitization profile.

DISCUSSION

The present study demonstrates the expression of the $\alpha 7$ nAChR subunit in denervated mouse muscle by RT-PCR. The $\alpha 7$ subunit has previously been observed in denervated rat muscle by immunohistochemistry and *in situ* hybridization (Fischer *et al.* 1999), and during muscle cell differentiation (Campos-Caro *et al.* 2001). Electrophysiological experiments presented here indicate that $\alpha 7^*$ nAChRs are functional and partially mediate nicotinic responses in the denervated muscle. This conclusion was based on the use of the selective agonist choline, after inhibiting muscle-type nAChRs with αCTxGI . The conclusion was further supported because this nicotinic activity was never present in $\alpha 7$ -null mice. Results obtained by comparing wild-type $\alpha 7^{+/+}$ and $\alpha 7^{-/-}$ mice indicate that there is functional nicotinic activity via $\alpha 7^*$ nAChRs in these non-neuronal cells.

We carried out intracellular recordings with rapid application of choline and ACh. Although choline is a full agonist for $\alpha 7^*$ nAChRs (Papke *et al.* 1996), the selectivity is not perfect. In fact, choline acts as a weak agonist for muscle-type, $\alpha 3\beta 4$, and $\alpha 4\beta 4$ nAChRs expressed in *Xenopus* oocytes (Papke *et al.* 1996; Alkondon *et al.* 1997; Zwart & Vijverberg, 2000). It has been reported that choline causes depolarization of muscle membranes (Del Castillo & Katz, 1957; Portela *et al.* 1970), and that the choline-evoked depolarization is enhanced after the muscle denervation (Portela *et al.* 1969; Fischer *et al.* 1999). Choline also increases the intracellular Ca^{2+} concentration at the muscle endplates (Dezaki *et al.* 1999). These earlier studies observed the choline-induced currents mediated mainly by muscle-type nAChRs. After inhibiting muscle-type nAChRs, we showed there is another choline-induced conductance in denervated muscle, and that conductance requires the presence of the $\alpha 7$ subunit. Although it is possible that the $\alpha 7$ subunit acts to help produce a conductance without participating in the actual nAChR

channel, our results strongly support the idea that functional $\alpha 7^*$ nAChRs are upregulated after muscle denervation.

We utilized αCTxGI , a muscle-type nAChR inhibitor, to determine that the choline-evoked depolarization from innervated muscles was almost exclusively mediated by junctional muscle-type nAChRs. Upon denervation, the subsequent nAChR supersensitivity observed with several nicotinic agonists (Cangiano, 1985) arises mainly from upregulation of the extrajunctional muscle nAChRs composed of $\alpha 1\beta 1\gamma\delta$ subunits (Hall & Sanes, 1993). The nicotinic activity on the surface of denervated muscle membrane was strongly diminished by αCTxGI at the same concentration (0.1 μM ; Fig. 3) used to inhibit the mouse extrajunctional nAChRs expressed in oocytes (Johnson *et al.* 1995) and the junctional nAChRs in mouse innervated muscle (Fig. 2). αCTxGI appears to have a similar potency at these two types of $\alpha 1$ -containing nAChRs expressed in muscle following denervation.

After inhibiting muscle-type nAChRs with αCTxGI in the denervated muscle, the remaining choline-evoked response arose from an $\alpha 7^*$ nAChR with an unusual pharmacology. Pancuronium was a less potent blocker of the αCTxGI -resistant depolarization (putative $\alpha 7^*$ nAChRs) than of the muscle-type, junctional nAChR response. The difference in the inhibition by F3 between muscle-type nAChRs and the nAChR type mediating the αCTxGI -resistant depolarization further supports the participation of $\alpha 7$. F3 is a ligand that inhibits $\alpha 7^*$ nAChRs with nanomolar affinity, but inhibits muscle-type nAChRs only at higher concentrations. The IC_{50} values of F3 for chick and rat $\alpha 7$ -homomeric nAChRs expressed in oocytes are 119 and 1 nM, respectively (Gotti *et al.* 1998, 2000), whereas the IC_{50} for muscle-type nAChRs is 3 μM (Dr C. Gotti, University of Milan, Italy, personal communication). These IC_{50} values of F3 for $\alpha 7$ nAChRs and muscle-type nAChRs are comparable to those obtained in this study for the αCTxGI -resistant response to choline (264 nM) and for the junctional nAChR response (1.6 μM), respectively.

The lack of inhibition by MLA, a potent $\alpha 7$ -homomeric nAChR antagonist in neurons, of the αCTxGI -resistant component is of considerable interest. No antagonism by MLA has been reported for the native $\alpha 7^*$ nAChR responses in chick ventral lateral geniculate nucleus (Guo *et al.* 1998; Guo & Chiappinelli, 2002), in chick sympathetic ganglion (Yu & Role, 1998*a,b*) and in rat dorsal motor nucleus (Ferreira *et al.* 2001). Furthermore, a recent paper describing a choline-sensitive nicotinic response in the lateral geniculate nucleus (Guo & Chiappinelli, 2002) may be relevant to our results, because both those responses persist during prolonged choline exposure, in contrast to

typical $\alpha 7$ receptors, which desensitize rapidly (Alkondon *et al.* 1997). Although the molecular basis for these atypical pharmacological properties is unknown, heteromeric $\alpha 7^*$ nAChRs have been proposed to explain the unique characteristics. It has been proposed that the native $\alpha 3\alpha 7^*$ nAChR expressed in chick sympathetic ganglion neurons is sensitive to α -BgTx but not to MLA, whereas an $\alpha 3\alpha 5\alpha 7^*$ nAChR is sensitive to MLA but not to α -BgTx (Yu & Role, 1998a,b). The $\alpha 7$ subunit can also co-assemble with muscle-type subunits to form $\alpha 7_2\beta 1\gamma\delta$ nAChRs under some conditions when expressed in *Xenopus* oocytes (Helekar *et al.* 1994), but in chick embryonic muscle the $\alpha 7$ subunit was not found in combination with $\alpha 1$, $\beta 1$ and δ subunits (Corriveau *et al.* 1995; Romano *et al.* 1997). Since several neuronal nAChR subunits ($\alpha 4$, $\beta 2$ and $\alpha 5$) have been found in mammalian muscle (Sala *et al.* 1996; Flora *et al.* 2000), there could be other possible combinations for heteromeric nAChRs. Accordingly, our data are consistent with denervated muscle expressing putative heteromeric $\alpha 7^*$ nAChR(s) with a low sensitivity to MLA.

Post-translational processing is another factor that could modulate the $\alpha 7^*$ nAChR function. Homomeric $\alpha 7$ nAChRs display multiple charged forms and different conformations in rat brain and in PC12 cells (Rakhilin *et al.* 1999; Drisdell & Green, 2000). These studies suggest that muscle-specific processing events may induce conformational changes in the $\alpha 7$ subunit that decrease the sensitivity to MLA. Alternatively, other more unlikely possibilities cannot be eliminated, such as the following: the α CTxGI-resistant effect of choline might be mediated by an unknown type(s) of nAChR(s) whose expression is regulated by the $\alpha 7$ gene product.

The functional significance of $\alpha 7^*$ nAChRs in the denervated muscle remains to be elucidated, but the following are reasonable speculations. Because $\alpha 7^*$ nAChRs have a much higher calcium permeability than muscle-type nAChRs (Decker & Dani, 1990; Vernino *et al.* 1992, 1994; Séguéla *et al.* 1993; Castro & Albuquerque, 1995), the activity-dependent Ca^{2+} signals arising from $\alpha 7^*$ nAChRs may have a role in the changes associated with denervation. After the breakdown of the motor nerve, the denervated muscle expresses extrajunctional nAChRs to a large extent, and exhibits supersensitivity to innervation by 'foreign' nerves (Cangiano, 1985). $\alpha 7^*$ nAChR activity may also contribute to the reception of chemical signals from the foreign nerves. Furthermore, Ca^{2+} influx through $\alpha 7^*$ nAChRs may induce a specific proteolytic activity, such as Ca^{2+} -activated neutral protease (CANP; Connold *et al.* 1986), and promote remodelling of the extracellular matrix molecules. This process could help to expedite the redistribution of molecules that form the postsynaptic transmembrane complex (see Daniels, 1997).

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