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Motor Nerve Terminal Outgrowth and Acetylcholine Receptors: Inhibition of Terminal Outgrowth by α-Bungarotoxin and Anti-Acetylcholine Receptor Antibody[1]

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Abstract

Motor nerves undergo extensive terminal outgrowth when the muscles they supply are "functionally denervated." In this study, we have investigated the role of the acetylcholine receptors (AChRs), newly appearing in such muscles, in promoting nerve terminal outgrowth. The amount of outgrowth was determined by morphometric measurement of (*a*) nerve terminal branching, (*b*) endplate length, and (*c*) ultraterminal sprouts, in cholinesterase-silver-stained neuromuscular junctions. Presynaptic neuromuscular blockade with botulinum toxin induced pronounced nerve terminal outgrowth in both the rat and mouse soleus muscles, although ultraterminal sprouts did not occur in the rat soleus. By contrast, postsynaptic neuromuscular blockade with α-bungarotoxin (α-BuTx) induced little or no terminal outgrowth, although it caused "functional denervation." Moreover, α-BuTx and anti-AChR antibody inhibited the terminal outgrowth otherwise induced by botulinum toxin. Other types of motor nerve growth, such as nerve regeneration, were unaffected by these agents. Our results are consistent with the concept that extrajunctional AChRs in skeletal muscle play an important role in the control of motor nerve terminal outgrowth at neuromuscular junctions.

Motor nerves normally undergo a continuous process of outgrowth (or "sprouting") of new branches from their terminal axonal arborizations (Barker and Ip, 1966; Tuffery 1971; Kemplay and Stolkin, 1980; Wernig et al., 1980; Smith and Rosenheimer, 1982). This process is greatly increased by treatment with pharmacological agents, such as botulinum toxin (Duchen and Stritch, 1968; Pestronk and Drachman, 1978a), that interfere with cholinergic transmission (Brooks, 1954) and produce denervation-like changes in muscle (Brown and Ironton, 1977; Pestronk and Drachman, 1978b). Previously, we have reported evidence that nerve terminal outgrowth is influenced by the increase of extrajunctional acetylcholine receptors (AChRs) that occurs in functionally denervated muscle (Pestronk and Drachman, 1978b). First, the degree of terminal outgrowth (nerve terminal branching and endplate elongation) correlates with levels of extrajunctional AChRs in the muscle membrane. Second, α-bungarotoxin (α-BuTx), a snake venom fraction that specifically blocks the functional sites of AChRs (Miledi and Potter, 1971; Berg et al., 1972; Lee 1972), significantly inhibits terminal outgrowth. However, the latter finding has been questioned in a study that used methods that differed in several respects from our own (Holland and Brown, 1980). In the present investigation we have re-examined the discrepancy in results by comparing the two experimental systems; in addition, we have used several new methods to study the effects of AChR blockade on nerve terminal outgrowth. The results now resolve the apparent differences and confirm and extend our previous findings. They are consistent with the idea that extrajunctional AChRs or a closely related molecule play an important role in the control of motor nerve terminal outgrowth.

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Materials and Methods

For these experiments, 46 female Sprague-Dawley rats and 24 female Lewis rats weighing 170 to 230 gm, and 135 female Swiss mice weighing 25 to 40 gm were used.

Staining of neuromuscular junctions

Soleus muscles were removed, pinned at resting length, and frozen in isopentane cooled with solid CO_2. The muscles were sectioned longitudinally (50-μm thickness for rats and 30-μm thickness for mice) in a cryostat. A combined cholinesterase-silver stain was used (Pestronk and Drachman, 1978a) that displays the cholinesterase-containing endplates as well demarcated transparent blue zones against which the silver-stained nerve terminals stand out clearly as black linear structures. This consistent staining method allows measurement of nearly all endplates in each section. The stained slides were coded and examined in a "blind" fashion, so that the microscopist had no knowledge of the experimental treatment performed on the muscle.

Measurement of terminal outgrowth

Nerve terminal outgrowth at the neuromuscular junction may be manifested by an increase in the number of nerve terminal branches ending within the endplate, by an increase in the length of the endplate, and by the occurrence of "ultraterminal" sprouts, new terminal branches that extend *beyond* the cholinesterase-stained endplate zone (Figs. 1, *a* and *b*, and 2, *a* to *f*).

To quantitate terminal outgrowth, we measured at least 20 endplates in each muscle by (*1*) counting the number of nerve terminal branch points within each cholinesterase-stained endplate area, (*2*) measuring the length of each endplate area parallel to the length of the muscle fiber, as outlined by the cholinesterase stain, and (*3*) counting the number of ultraterminal sprouts that originated from each endplate. The frequency of ultraterminal sprouting was expressed in terms of: (*a*) the proportion of endplates with ultraterminal sprouts and (*b*) the average number of ultraterminal sprouts per endplate. Each measurement has proved reliable and reproducible. Similar results were found when repeated "blind" counts were made by the same or different examiners. The endplate length measured on the basis of the cholinesterase stain was equal to measurements obtained using immunohis-

tochemical staining of postsynaptic junctional AChRs (W. C. Yee and A. Pestronk, unpublished observations). These measures of endplate length closely reflect the length of the nerve terminal arborization as demonstrated by the silver stain.

Botulinum toxin treatment

Crystalline type A botulinum toxin, freshly diluted in mammalian Ringer's solution (1.2×10^{-9} gm in 30 μl for the rat and 1.2×10^{-10} gm in 10 μl for the mouse) was injected directly into the surgically exposed soleus muscle, with the use of a fine 30 gauge needle.

α-BuTx treatment

α-BuTx obtained from the Miami Serpentarium was repurified by ion exchange chromatography using Whatman CM-32 columns prior to use.

Injection. Animals were treated with daily injections of α-BuTx in Ringer's solution (3 μg in 30 μl in rats or 1 μg in 10 μl in mice) into the soleus muscle. Using this method of α-BuTx treatment, initial AChR blockade is initially complete as measured by lack of miniature endplate potentials of muscle response to nerve stimulation and of further α-BuTx binding. However, partial recovery of the AChR blockade occurs in the 24-hr period between injection (see also Chang et al., 1975; Fertuck et al., 1975; Pestronk and Drachman, 1978b). Control muscles received daily injections of 0.5 to 1.0 μg of bovine serum albumin in a similar volume of Ringer's solution.

Perfusion. Soleus muscles were treated continuously with α-BuTx by use of implantable osmotic *pumps*. At the beginning of each experiment a loading dose of α-BuTx in Ringer's solution (3 μg in 30 μl for rats or 0.6 μg in 10 μl for mice) was injected into the soleus muscle through a fine 30 gauge needle. Neuromuscular blockade was then maintained by continuous infusion of α-BuTx in Ringer's solution (0.2 mg/ml at 1 μl/hr for rats and 0.06 mg/ml at 0.47 μl/hr for mice) by means of Alzet osmotic infusion pumps implanted subcutaneously in the abdominal wall. The α-BuTx solution was delivered directly over the body of the soleus muscle via tapered, flexible polyethylene tubing sutured in place. Control animals had pumps and tubing filled with Ringer's solution sutured in a similar position. After 7 days the muscles were removed, and nerve terminal outgrowth was evaluated as described above.

Perfusion of α-BuTx over the soleus muscle produced persistent and complete blockade of AChRs throughout the experimental period measured as above. In addition, this method of α-BuTx treatment resulted in more denervation changes in muscle. In perfused muscles, levels of extrajunctional AChRs (measured by the binding of [^{125}I]-α-BuTx) averaged 95 $\pm$ 8% of those seen after denervation, significantly ($p < 0.05$) more than the 62 $\pm$ 12% found with intermittent α-BuTx injections (A. Pestronk, unpublished observations). Thus, perfusion of α-Butx is more effective than intermittent injections in maintaining AChR blockade.

Reinnervation of endplates after nerve crush

In these studies, nerve regeneration was induced by crushing the nerve to the soleus at its point of entry into the muscle, by means of a pair of fine jeweler's forceps. The effectiveness of axonal regeneration was evaluated by measuring the percentage of old synaptic sites (cholinesterase-stained zones) reinnervated at 7 to 14 days after the nerve to the soleus was crushed. Terminal outgrowth of the regenerated axons at the reinnervated endplates was also measured, as described above.

Passive transfer of human anti-AChR antibody

Mice were treated with serum from a human patient with myasthenia gravis. One milliliter of serum from a patient with myasthenia gravis (anti-human AChR antibody titer 121×10^{-9} M) was injected intraperitoneally into each mouse daily for 9 days. Sprouting experiments were begun 2 days after the initial serum injection. Control animals received normal human serum injected daily in the same manner.

Antibodies to AChR in rats with experimental autoimmune myasthenia gravis (EAMG)

AChR was purified by affinity chromatography (Eldefrawi and Eldefrawi, 1973) from the electric organ of *Torpedo californicus*. Female Lewis rats (175 to 200 gm) were immunized with 50 μg of AChR, emulsified in an equal volume of Freund's complete adjuvant, injected intradermally.

Measurement of anti-AChR antibodies was carried out by an immunoprecipitation assay similar to those previously described (Pestronk et al., 1983). All immunized rats had strongly positive anti-AChR antibody titers 4 weeks after immunization at the time of the sprouting experiments: anti-*Torpedo* AChR titers averaged $1036 \pm 205 \times 10^{-9}$ M on day 0, and $1459 \pm 197 \times 10^{-9}$ M on day 7. Anti-rat AChR titers averaged $14 \pm 4 \times 10^{-9}$ M on day 0, and $47 \pm 17 \times 10^{-9}$ M on day 7.

Results

Nerve terminal outgrowth induced by botulinum toxin treatment (Table I)

Rat. Seven days after treatment with botulinum toxin there was a significant amount of nerve terminal outgrowth in rat soleus muscles (Fig. 1, *a* and *b*). Nerve terminal branch points increased by an average of 1.4/endplate, and endplate length increased by 12 μm. No ultraterminal sprouting was present.

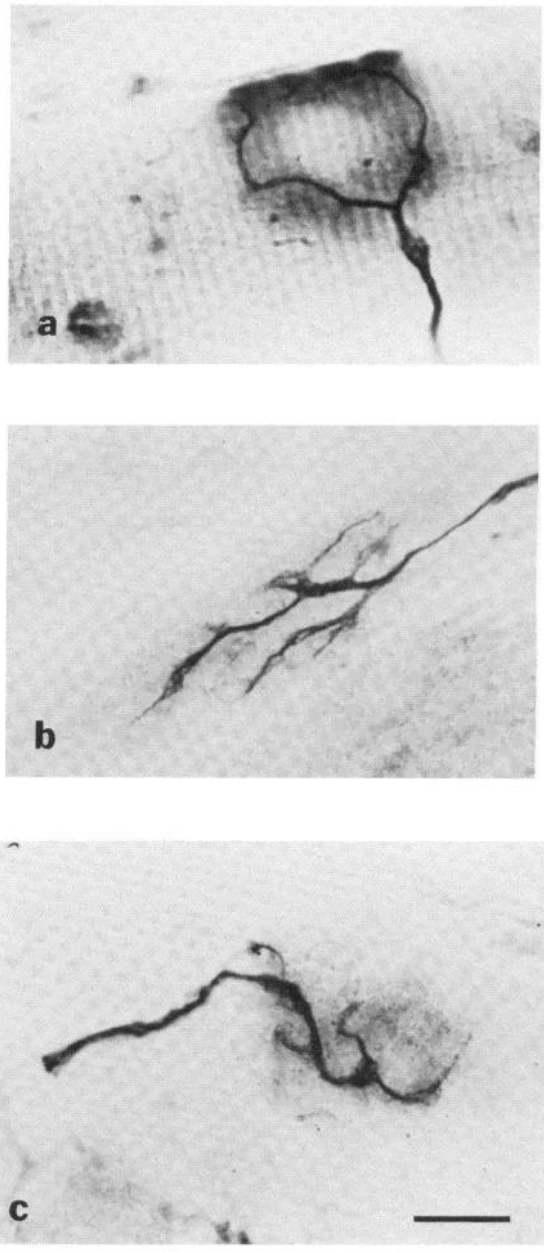

Figure 1. Cholinesterase-silver-stained endplates from rat soleus muscles. *a*, Endplate from a normal muscle. Endplate length is short and there are few branch points. *b*, Endplate from a muscle 7 days after treatment with botulinum toxin. There is significant terminal sprouting with increased endplate length and nerve terminal branching but no ultraterminal sprouts. *c*, Endplate from a muscle treated with α-BuTx for 7 days. There is no significant change from controls in endplate length or in terminal branching. *Bar* = 20 μm.

TABLE I

Effects of botulinum toxin on nerve terminal outgrowth

Table I gives a comparison of terminal outgrowth in rat and mouse soleus muscles treated with botulinum toxin (Bot). Branch points and endplate length increased to a similar extent in the rat and the mouse, but ultraterminal sprouts increased only in the mouse. Results are expressed as mean ± SE.

Sprouting Characteristic	Rat		Mouse	
	Control (7)[a]	Bot (6)	Control (15)	Bot (16)
Branch points/endplate	3.2 ± 0.3	4.6 ± 0.2[b]	2.7 ± 0.1	4.2 ± 0.1[b]
Increase		1.4		1.5
Endplate length (μm)	40 ± 4	52 ± 6[b]	43 ± 1	55 ± 2[b]
Increase		12		12
Ultraterminal sprouts/endplate	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.01	0.71 ± 0.08[b]
Increase		0.0		0.68
Endplates with ultraterminal sprouts	0.02 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.46 ± 0.04[b]

[a] Numbers in parentheses, number of muscles sampled.

[b] Greater than control, $p < 0.001$.

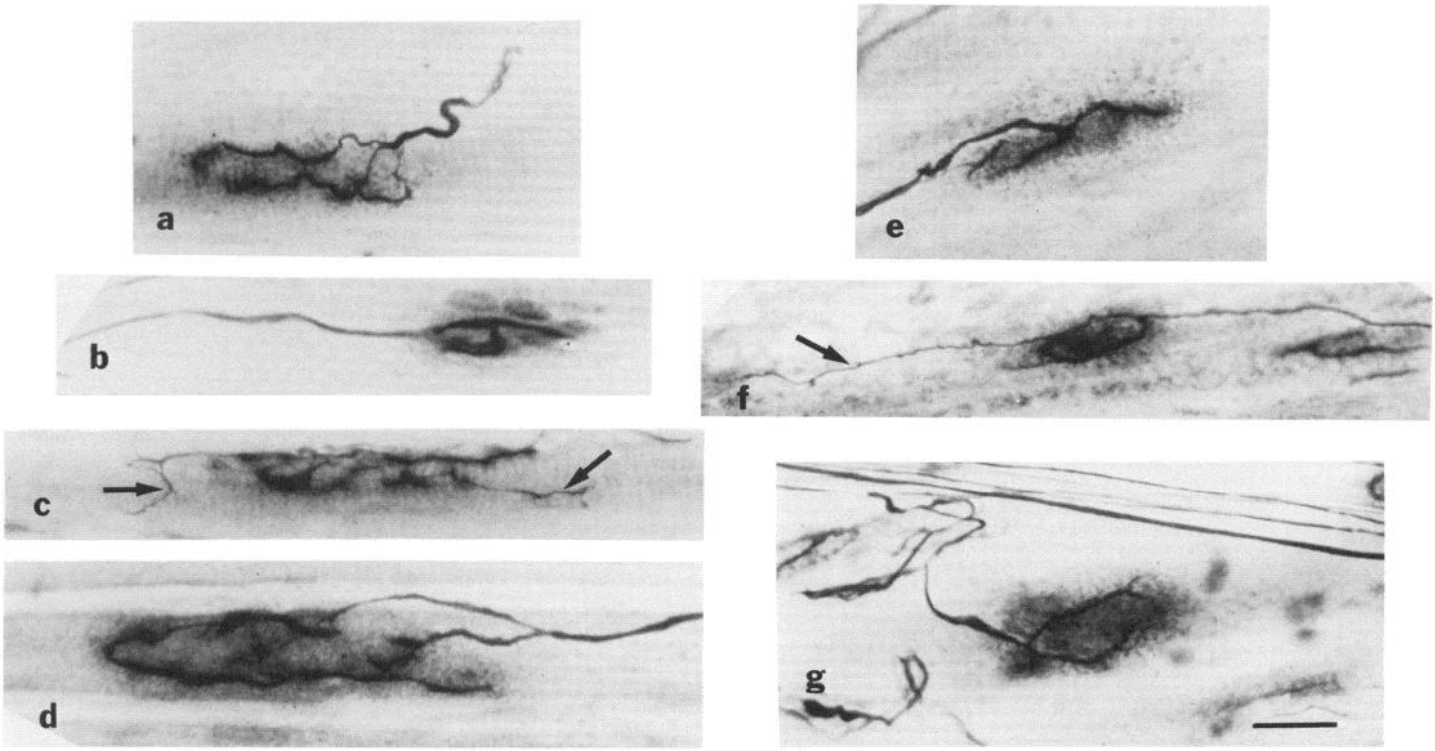

Figure 2. Cholinesterase-silver-stained endplates from mouse soleus muscles. *a* and *b*, Endplates from normal muscles. *c*, Endplate from muscle 7 days after treatment with botulinum toxin. Several ultraterminal sprouts are present (*arrows*). *d*, Endplate from a muscle 7 days after treatment with botulinum toxin with increased endplate length and nerve terminal branching. *e*, Endplate from a muscle treated with α-BuTx for 7 days. *f*, Endplate from a muscle treated with botulinum and α-BuTx injections. Terminal branching and endplate length are normal but a long ultraterminal sprout is present. *g*, Endplate from a muscle treated with botulinum and continuous α-BuTx perfusion. There is no increase in endplate length or terminal branching. *Bar* = 20 μm.

Mouse. As in the rat, botulinum toxin induced a marked change in the mean number of nerve terminal branch points, with an increase of 1.5/endplate. The endplate length also increased significantly by 12 μm. However, in contrast to the rat soleus, where no ultraterminal terminal sprouts were detected (Table I), the mouse soleus showed an increase of ultraterminal sprouts, averaging 0.68/endplate (see Fig. 2, *a* to *d*).

Nerve outgrowth induced by α-BuTx treatment (Table II)

In the rat soleus there was no significant terminal outgrowth after 7 days of α-BuTx injections (Fig. 1*c*). Endplate length and nerve terminal branching did not change significantly, and no ultraterminal sprouting was seen (Table II). In the mouse soleus (Fig. 2*e*), 7 days of injection or perfusion of α-BuTx produced a small amount of nerve terminal sprouting. This was manifested by a slight increase in terminal branch points (0.2 to 0.5 branch point/endplate) and ultraterminal sprouts (0.06 to 0.16 ultraterminal sprout/endplate) without a significant change in endplate length (Table II).

Effect of α-BuTx on nerve terminal outgrowth induced by botulinum toxin (Table III)

In a previous study we found that injection of α-BuTx produces virtually complete inhibition of the terminal outgrowth otherwise seen in botulinum-treated rat soleus muscle. In the present study, our results show that α-BuTx also significantly inhibits terminal outgrowth in the *mouse* soleus muscle and that continuous infusion produces a greater effect than intermittent daily injection. In botulinum-treated mouse soleus muscles *injected* daily with α-BuTx, increases in terminal branching and endplate length were inhibited by 44% and 77%, respectively, when compared to botulinum-treated muscles

TABLE II

Effects of α-BuTx on nerve terminal outgrowth

Soleus muscles were treated for 7 days with α-BuTx. α-BuTx induced no terminal outgrowth in the rat and only a minor degree of outgrowth in the mouse.

Sprouting Characteristic	Rat		Mouse		
	Control (7)[a]	α-BuTx Injection (7)	Control (15)	α-BuTx Injection (5)	α-BuTx Pump (8)
Branch points/endplate	3.2 ± 0.2[b]	3.3 ± 0.2	2.7 ± 0.1	2.9 ± 0.1	3.2 ± 0.2[b]
Increase		0.1		0.2	0.5
Endplate length (μm)	39 ± 2	40 ± 2	43 ± 1	44 ± 1	42 ± 3
Increase		1.0		1	−1
Ultraterminal sprouts/endplate	0.02 ± 0.01	0.02 ± 0.02	0.03 ± 0.01	0.19 ± 0.07[b]	0.09 ± 0.02[c]
Increase		0.0		0.16	0.06
Endplates with ultraterminal sprouts	0.02 ± 0.01	0.02 ± 0.02	0.02 ± 0.01	0.14 ± 0.05[b]	0.07 ± 0.02[c]

[a] Numbers in parentheses, number of muscles sampled.
[b] Greater than control, $p < 0.05$.
[c] Greater than control, $p < 0.02$.

TABLE III

Effects of α-BuTx on botulinum-induced terminal outgrowth in the mouse

Mouse soleus muscles were treated with botulinum toxin (Bot) on day 0. α-BuTx injections or perfusions were maintained for 7 days, as indicated. Note (*a*) that α-BuTx inhibited the botulinum-induced nerve terminal outgrowth, and (*b*) that perfusion of α-BuTx was more effective than injection, especially in inhibiting ultraterminal sprouts.

Sprouting Characteristic	Injection			Perfusion		
	Control (8)[a]	Bot (8)	Bot + α-BuTx (5)	Control (8)	Bot (8)	Bot + α-BuTx (9)
Branch points/endplate	2.7 ± 0.1	4.3 ± 0.2[b]	3.6 ± 0.3[b,c]	2.7 ± 0.1	4.1 ± 0.2[b]	3.2 ± 0.2[d,e]
Increase		1.6	0.9		1.4	0.5
Endplate length (μm)	45 ± 1	57 ± 3[b]	48 ± 3[c]	41 ± 1	53 ± 1[b]	44 ± 1[d]
Increase		12	3		12	3
Ultraterminal sprouts/endplate	0.04 ± 0.01	0.73 ± 0.11[b]	0.72 ± 0.17[f]	0.03 ± 0.01	0.69 ± 0.11[b]	0.17 ± 0.09[d,g]
Increase		0.69	0.68		0.66	0.14
Endplates with ultraterminal sprouts	0.02 ± 0.01	0.45 ± 0.05[b]	0.49 ± 0.10[f]	0.02 ± 0.01	0.47 ± 0.07[b]	0.12 ± 0.05[d,g]

[a] Numbers in parentheses, number of muscles sampled.
[b] Greater than control, $p < 0.001$.
[c] Less than botulinum, $p < 0.05$.
[d] Less than botulinum, $p < 0.001$.
[e] Greater than control, $p < 0.05$.
[f] Greater than control, $p < 0.005$.
[g] Less than botulinum and botulinum + injected α-Butx, $p < 0.005$.

injected daily with albumin (Table III), but there was no effect on ultraterminal sprouting (Fig. 2*f*). By contrast, *continuous perfusion* of the mouse soleus with α-BuTx from implanted pumps inhibited all three forms of terminal outgrowth (Table III, Fig. 2*g*). Botulinum-induced increases in terminal branch points, endplate length, and ultraterminal sprouting were significantly inhibited by 64%, 76%, and 79% respectively ($p < 0.001$).

Effect of α-BuTx on regeneration following nerve crush

To determine whether α-BuTx had an inhibitory effect on nerve outgrowth per se, we examined its effect on axonal regeneration. Axons regenerating after a crush injury tend to grow until they reinnervate muscle fibers at or near the original synaptic sites. To study the effect of α-BuTx on the ability of axons to grow, we crushed the nerves to the soleus in two groups of mice and treated one group with continuous local α-BuTx infusion. At 2 days after nerve crush, no axons were visible within the muscles in either group, indicating that the crush procedure produces early complete loss of intramuscular axons. By 7 days, 76 ± 6% of the original endplates had been reinnervated in nine control animals. This figure was not significantly changed (73 ± 4%) in the soleus muscles of the nine mice perfused with α-BuTx during the 7-day regeneration period, indicating that α-BuTx does not inhibit axonal regeneration.

Terminal outgrowth of regenerating axons at old synaptic sites (Table IV)

When regenerating axons make contact with muscle fibers, they undergo terminal branching and an increase in the area of synaptic contact. Because this pattern of outgrowth appears very similar to the terminal outgrowth induced by botulinum toxin, we examined the effects of α-BuTx on this process.

In control untreated soleus muscles of 3-month-old rats, endplate length averaged 38 ± 1 μm and nerve terminals had 2.8 ± 0.1 branch points. In soleus muscles reinnervated after nerve crush 14 days previously, 91% of the muscle fibers were reinnervated. Single fibers with two areas of cholinesterase staining were rare, suggesting that reinnervation occurred at the site of the original synapses. However, there were significant changes in endplate morphology in reinnervated muscles compared with controls. Reinnervated endplates were enlarged. The nerve terminals themselves appeared thin, and their terminal branching was considerably less profuse than before nerve crush (Table IV).

To examine the effect of α-BuTx blockade of AChRs on the terminal sprouting occurring during reinnervation, we injected α-BuTx daily into the soleus muscles of rats for the first 7 days after nerve crush. Our results showed that, after 14 days, 90% of muscle fibers were reinnervated, which again did not differ from reinnervation

in control muscles. However, α-BuTx eliminated the expansion of endplate length that occurs normally with reinnervation. In the α-BuTx-treated reinnervated muscles, both endplate length and the amount of terminal branching were significantly less in than in control reinnervated muscles (Table IV).

Effects of human anti-AChR antibody on terminal outgrowth (Table V)

The inhibition of nerve terminal outgrowth by α-BuTx suggests that AChRs may be involved in this process. To test this idea further, we studied the effect of serum known to have a high titer of anti-AChR antibody, from a human with myasthenia gravis, on terminal sprouting in the mouse (Table V). Nine days of treatment with myasthenic serum had no effect on the morphology of nerve terminals in normal animals. Nerve terminal branching, endplate length, and ultraterminal sprouts were similar in animals given myasthenic serum and normal control human serum. In botulinum-treated mice given daily injections of myasthenic serum there was a significant inhibition of terminal outgrowth. The botulinum toxin-induced increase in branch points and endplate length was inhibited by approximately 50% ($p < 0.005$) compared with control serum-treated animals. However, the myasthenic serum treatment had no significant effect on ultraterminal sprouting (Table V).

Effects of immunization with AChR on nerve terminal outgowth (Table VI)

We next examined nerve terminal outgrowth in rats which had been immunized with purified *Torpedo* AChR and therefore had high titers of anti-AChR antibody (Table VI). In rats immunized with AChR, soleus endplates were somewhat larger and more complex than in normal rats. Seven days after injection of botulinum toxin, endplate length was unchanged and there was a small but not significant increase in terminal branch points. By contrast, non-EAMG Lewis rats developed marked nerve terminal outgrowth after botulinum treatment (Table VI). Seven days after botulinum treatment the mean endplate length increased by 12 μm and terminal branch points increased by 1.3/endplate in the soleus muscles.

Discussion

Denervation changes in muscle and nerve terminal outgrowth. Denervation changes in skeletal muscle play an important role in eliciting nerve terminal outgrowth at the neuromuscular junction (Duchen and Stritch, 1968; Brown and Ironton, 1977; Pestronk and Drachman, 1978b). Two lines of evidence support this idea. (*1*) The amount of nerve terminal sprouting correlates with the degree of denervation change in the muscle (Pestronk and Drachman, 1978b). In previous experiments, functional denervation of skeletal muscle was produced by (*a*) presynaptic blockade of acetylcholine (ACh) transmission by botulinum toxin (Brooks, 1954; Tonge, 1974; Pestronk and Drachman, 1976) or (*b*) tetrodotoxin blockade of nerve impulse transmission (muscle disuse) (Lavoie et al., 1976; Pestronk et al., 1976). Our results showed that there was a significant correlation between the amount of terminal outgrowth, measured by increased terminal branching and endplate length, and the degree of denervation changes, as indicated by levels of extrajunctional AChRs (Pestronk and Drachman, 1978b). (*2*) When muscle denervation changes are inhibited, the amount of terminal outgrowth is correspondingly reduced. For example, Duchen and Tonge (1977) reduced muscle denervation changes by implanting a foreign (non-poisoned) nerve into botulinum-treated muscles. As a result, the terminal outgrowth that normally occurs after botulinum toxin treatment was inhibited (Duchen and Tonge, 1977). Brown et al. (1980) used direct electrical stimulation to reduce levels of denervation changes (extrajunctional ACh sensitivity) in botulinum-treated muscles. The resulting reduction of denervation changes inhibited the appearance of terminal outgrowth. These studies all lead to the conclusion that a stimulus for terminal outgrowth arises from functionally denervated muscle fibers.

TABLE IV

Terminal outgrowth at reinnervated endplates

Fourteen days were allowed for regeneration after nerve crush at its point of entry into the rat soleus muscle. α-BuTx-treated muscles were injected daily for 7 days after the nerve crush. Note that α-BuTx inhibited nerve terminal branching and endplate elongation but did not interfere with reinnervation.

Sprouting Characteristic	Control (7)[a]	Reinnervated (7)	Reinnervated + α-BuTx (5)
Branch points/endplate	2.8 ± 0.1	1.9 ± 0.1[b]	1.6 ± 0.1[c]
Endplate Length (μm)	38 ± 1	45 ± 1[b]	40 ± 1[d]
Percentage of endplates innervated	98 ± 1	91 ± 2[b]	90 ± 2[b]

[a] Numbers in parentheses, number of muscles sampled.
[b] Different from control, $p < 0.001$.
[c] Less than reinnervated, $p < 0.02$.
[d] Less than reinnervated, $p < 0.002$.

AChRs and nerve terminal outgrowth. The appearance of new AChRs in muscle membrane following denervation may itself play an important role in eliciting nerve terminal outgrowth. First, as noted above, the extent of nerve terminal outgrowth correlates with the level of increase of extrajunctional AChRs in muscle membrane (Pestronk and Drachman, 1978b). More direct support comes from our finding that α-BuTx, which specifically blocks AChRs, also inhibits nerve terminal outgrowth (Pestronk and Drachman, 1978b). The expected increase in endplate size and nerve terminal branching in botulinum-treated muscles was largely inhibited when the muscles were also treated with α-BuTx. Other studies have found similar effects of AChR blocking agents on the terminal outgrowth that occurs during development. The size and complexity of endplates in developing chick embryos is greatly restricted by treatment *in ovo* with α-BuTx or α-cobratoxin (Giacobini et al., 1973; Freeman et al., 1976). Curare treatment reduces the number of small terminal sprouts at chicken embryo endplates (Ding et al., 1983). The present results confirm and extend these observations. Our data show again that α-BuTx significantly inhibits nerve terminal outgrowth. In addition, we have found that terminal outgrowth is reduced in the presence of other agents that interact with AChRs, including serum containing anti-AChR antibody from a myasthenia gravis patient and in animals specifically immunized against AChR.

The inhibitory effect of α-BuTx on nerve terminal outgrowth has been disputed by Holland and Brown (1980). In their experiments ultraterminal sprouting was evaluated histologically in mice that were first treated with botulinum toxin to induce sprouting and then were injected with α-BuTx. Their results failed to show inhibition of ultraterminal sprouting by α-BuTx.

Our new findings show that the apparent discrepancies in results are attributable to three differences in the experimental procedures used: (*1*) the species of experimental animal, (*2*) the criteria for measurement of terminal outgrowth, and (*3*) the method of administration of α-BuTx. In the rat soleus muscle, nerve terminal outgrowth after botulinum treatment is manifested by increased terminal branching and elongation of the endplates, but not by ultraterminal sprouting. In the mouse, all three forms of nerve terminal outgrowth occur. In our study, we examined all three manifestations of terminal outgrowth, whereas Holland and Brown (1980) relied solely on ultraterminal sprouting. In addition, the method of administration of α-BuTx makes an important difference both in the degree of blockade of AChRs (see "Materials and Methods") and in the inhibition of terminal outgrowth. Our results show that intermittent injection of α-BuTx (as carried out by Holland and Brown (1980)) produces at most a partial reduction of terminal outgrowth; terminal branching and endplate elongation are more sensitive to this treatment, whereas ultraterminal sprouts in mice, the sole measure of terminal outgrowth used by Holland and Brown (1980), may not be affected at all by incomplete α-BuTx blockade. In the mouse, α-BuTx must

TABLE V

Effects of human anti-AChR antibody on terminal outgrowth

Soleus muscles from mice were treated for 9 days with serum from myasthenic humans with myasthenia gravis or from control individuals. Botulinum toxin was injected on day 2. The myasthenic serum significantly inhibited the increase of nerve terminal branch points and endplate length but did not interfere with ultraterminal sprouting.

	Control Serum		Myasthenic Serum	
	Untreated (8)[a]	Botulinum (10)	Untreated (8)	Botulinum (7)
Branch points/endplate	2.7 ± 0.1	4.1 ± 0.2[b]	2.7 ± 0.1	3.4 ± 0.2[b,c]
Increase		1.4		0.7
Endplate length (μm)	39 ± 1	47 ± 1[b]	39 ± 1	43 ± 1[b,c]
Increase		8		4
Ultraterminal sprouts/endplate	0.02 ± 0.01	0.61 ± 0.09[b]	0.04 ± 0.01	0.54 ± 0.10[b]
Increase		0.59		0.50
Endplates with ultraterminal sprouts	0.02 ± 0.01	0.45 ± 0.06[b]	0.04 ± 0.01	0.42 ± 0.07[b]

[a] Numbers in parentheses, number of muscles sampled.
[b] Greater than untreated, $p < 0.001$.
[c] Less than control botulinum, $p < 0.005$.

TABLE VI

Effects of EAMG on botulinum-induced terminal outgrowth

Experiments were performed on rats with EAMG 4 to 5 weeks after immunization with AChR. Botulinum toxin was injected into the soleus muscle 7 days before the study. Note the inhibition of nerve terminal outgrowth in the rats with EAMG.

	Control		EAMG	
	Untreated (5)[a]	Botulinum (7)	Untreated (7)	Botulinum (5)
Branch points/endplate	3.3 ± 0.2	4.6 ± 0.1[b]	3.7 ± 0.1	4.1 ± 0.1[c]
Increase		1.3		0.4
Endplate length (μm)	41 ± 2	53 ± 2[b]	47 ± 1[d]	49 ± 2
Increase		12		2
Ultraterminal sprouts/endplate	0.02 ± 0.01	0.03 ± 0.01	0.04 ± 0.02	0.04 ± 0.02
Increase		0.01		0.0
Endplates with ultraterminal sprouts	0.02 ± 0.01	0.03 ± 0.01	0.04 ± 0.02	0.04 ± 0.02

[a] Numbers in parentheses, number of muscles sampled.
[b] Greater than control untreated, $p < 0.001$.
[c] Less than control botulinum, $p < 0.05$.
[d] Greater than control untreated, $p < 0.01$.

be applied by *continuous perfusion* directly over the soleus muscle in order to produce a maximal effect on terminal outgrowth. In contrast, the main changes in the botulinum-treated rat soleus, terminal branching and endplate elongation, are sensitive to even partial AChR blockade produced by daily α-BuTx injections (Pestronk and Drachman, 1978b; present results). The present data show that continuous α-BuTx blockade of AChRs by perfusion produces inhibition of outgrowth in both the rat and the mouse that is observable with any method of analysis. With more complete AChR blockade produced by α-BuTx perfusion, all three measures of nerve terminal outgrowth are inhibited by more than 70% even in the mouse.

There are at least three theoretically possible explanations for the ability of α-BuTx and anti-AChR antibody to inhibit nerve terminal outgrowth. First, these agents might reduce denervation changes in muscle, which would then not be sufficient or appropriate to evoke terminal outgrowth. This possibility is excluded, since α-BuTx actually *increases* denervation changes seen after botulinum treatment (Mathers and Thesleff, 1978; Drachman et al., 1982). Second, α-BuTx and anti-AChR antibody might *directly* damage the nerve or inerfere with its ability to grow. However, there are no pathological changes in the light microscopic or ultrastructural morphology of nerve terminals (or the postsynaptic apparatus) chronically treated with α-BuTx (Drachman et al., 1982) or human myasthenic serum (Toyka et al., 1978). In addition, neither α-BuTx nor anti-AChR antibody impairs the ability of the nerve to undergo other types of outgrowth such as collateral sprouting (A. Pestronk, manuscript in preparation) or regeneration (see above), even in the same muscles where terminal sprouting is inhibited. These findings appear to exclude the possibility of general sprout-inhibiting effects of α-BuTx and anti-AChR antibody. The third possible explanation seems most likely: i.e., that the inhibitory effects of α-BuTx and anti-AChR antibody on terminal sprouting result from their common actions on AChRs. Treatment with α-BuTx (a purified polypeptide snake toxin of $M_r = 8000$), passive transfer of human myasthenic immunoglobulin, and immunization of rats with AChR are very different procedures, but they share the ability to interfere specifically with AChRs. Possible mechanisms for their effects include blockade or steric hindrance of the ACh recognition site itself, steric hindrance of a nearby site, or some as yet undefined secondary consequence of AChR blockade. Further studies will be required to determine whether these mechanisms operate by interrupting an interaction between new nerve terminal branches and the surface components of the muscle fiber or, less likely, via secondary changes induced in muscle metabolism.

Dual and opposite effects of α-BuTx on nerve terminal outgrowth. Paradoxically, agents that interact directly with the AChR should have dual and opposite effects on terminal outgrowth at the neuromuscular junction. Their ability to inhibit transmission at the neuromuscular junction produces denervation changes in muscle (Edwards, 1979; Fambrough, 1979) (including an increase of extrajunctional AChRs). This in turn *elicits* terminal outgrowth. However, by binding to and thereby reducing available AChRs, these agents also tend to *inhibit* terminal outgrowth. Our results suggest that these

opposite effects are probably occurring simultaneously, and are competing. When mouse soleus muscles are treated with α-BuTx, denervation changes occur (i.e., an increase of extrajunctional AChRs), resulting in a small amount of terminal outgrowth. However, quantitative analysis shows that terminal outgrowth in these muscles is less than one would expect for the degree of denervation changes induced by α-BuTx, a consequence of its growth-inhibiting effects. A similar phenomenon is seen in the animals immunized against AChR. One month after immunization some terminal outgrowth has occurred. However, the terminal outgrowth (terminal branching and endplate enlargement) normally seen after botulinum treatment is inhibited. In this case it appears that an immune response to AChR both stimulates (see also Bickerstaff and Woolf, 1960) and inhibits terminal outgrowth.

Other factors influencing nerve terminal sprouting. The conclusion that AChRs are important in evoking nerve terminal outgrowth does not exclude the possibility that other factors may play a role as well. If the basal lamina from a prior synaptic site is present, AChR may not be necessary to initiate terminal outgrowth in regenerating nerves (Marshall et al., 1977). The characteristics of "synaptic" basal lamina that evoke terminal outgrowth from regenerating nerves are not known. However, when nerve terminal outgrowth occurs into areas without prior synaptic contact, such as during development, after botulinum treatment, or in enlargement of the endplate by regenerating axons (see "Terminal Outgrowth of Regenerating Axons at Old Synaptic Sites"), AChRs may play an important role.

The idea that trophic or chemical factors may evoke nerve terminal outgrowth has also been considered. At this time there is no direct evidence that any such substances stimulate *nerve terminal outgrowth in vivo*, where local or surface factors such as AChRs and the basal lamina seem to be important. Interestingly, an antibody against an $M_r = 56{,}000$ protein secreted by muscle has been found to inhibit ultraterminal sprouting (Gurney, 1984). There is some evidence that diffusible factors from muscle may play a role in initiating or directing another type of outgrowth, *collateral sprouting* (Ramón y Cajal, 1928; Hoffman, 1950; Hoffman and Springell, 1951; Diamond et al., 1976; O'Brien et al., 1978), since it must be evoked over a distance. Further information in this area awaits identification and characterization of all the factors that may stimulate or inhibit nerve outgrowth *in vivo*.

In any event, study of the control of terminal outgrowth and other forms of nerve growth must take into account the possible multifactorial nature of the process, including the intrinsic ability of the nerve to grow (Ruffolo et al., 1978; Black and Lasek, 1979; Pestronk et al., 1980; Huang and Keynes, 1983). Each type of motor nerve growth should be analyzed separately to determine the factors controlling it. Examination of the various types of nerve growth, and the similarities and differences between them, should contribute to a more precise understanding of neuronal sprouting and plasticity.

References

Barker, D., and M. C. Ip (1966) Sprouting and degeneration of mammalian motor axons in normal and deafferented skeletal muscle. Proc. R. Soc. Lond. (Biol.) *163:* 538–554.

Berg, D. K., R. B. Kelly, P. B. Sargent, P. Williamson, and W. Hall (1972) Binding of bungarotoxin to acetylcholine receptors in mammalian muscle. Proc. Natl. Acad. Sci. U. S. A. *69:* 147–151.

Bickerstaff, E. R., and A. L. Woolf (1960) The intramuscular nerve endings in myasthenia gravis. Brain *83:* 10–23.

Black, M. M., and R. J. Lasek (1979) Slowing of the rate of axonal regeneration during growth and maturation. Exp. Neurol. *63:* 108–119.

Brooks, V. B. (1954) The action of botulinum toxin on motor-nerve filaments. J. Physiol. (Lond.) *123:* 501–515.

Brown, M.C., and R. Ironton (1977) Motor nerve sprouting induced by prolonged tetrodotoxin block of nerve action potentials. Nature *265:* 459–461.

Brown, M. C., R. L. Holland, and R. Ironton (1980) Nodal and terminal sprouting from motor nerves in fast and slow muscles of the mouse. J. Physiol. (Lond.) *306:* 493–510.

Chang, C. C., S. -T. Chaung, and M. C. Huang (1975) Effects of chronic treatment with various neuromuscular blocking agents on the number and distribution of acetylcholine receptors in the rat diaphragm. J. Physiol. (Lond.) *250:* 161–173.

Diamond, J., E. Cooper, C. Turner, and L. Macintyre (1976) Trophic regulation of nerve sprouting. Science *193:* 371–377.

Ding, R., J. K. S. Jansen, N. G. Laing, and H. Tonnesen (1983) The innervation of skeletal muscles in chickens curarized during early development. J. Neurocytol. *12:* 887–919.

Drachman, D. B., E. F. Stanley, A. Pestronk, J. W. Griffin, and D. L. Price (1982) Neurotrophic regulation of two properties of skeletal muscle by impulse-dependent and spontaneous acetylcholine transmission. J. Neurosci. *2:* 232–243.

Duchen, L. W., and S. J. Strich (1968) The effects of botulinum toxin on the pattern of innervation of skeletal muscle in the mouse. Q. J. Exp. Physiol. *53:* 84–89.

Duchen, L. W., and D. A. Tonge (1977) The effects of implantation of an extra nerve on axonal sprouting usually induced by botulinum toxin in skeletal muscle of the mouse. J. Anat. *124:* 205–215.

Edwards, C. (1979) The effects of innervation on the properties of acetylcholine receptors in muscle. Neuroscience *4:* 565–584.

Eldefrawi, M. E., and A. T. Eldefrawi (1973) Purification and molecular properties of the acetylcholine receptor from *Torpedo electroplax*. Arch. Biochem. Biophys. *159:* 362–373.

Fambrough, D. M. (1979) Control of acetylcholine receptors in skeletal muscle. Physiol. Rev. *59:* 165–227.

Fertuck, H. C., W. Woodward, and M. M. Salpeter (1975) *In vivo* recovery of muscle contraction after α-bungarotoxin binding. J. Cell Biol. *66:* 209–213.

Freeman, S. S., A. G. Engel, and D. B. Drachman (1976) Experimental acetylcholine blockade of the neuromuscular junction. Effects on endplate and muscle fiber ultrastructure. Ann. N. Y. Acad. Sci. *274:* 44–59.

Giacobini, G., G. Filogamo, M. Weber, P. Boquet, and J. P. Changeux (1973) Effects of a snake α-neurotoxin on the development of innervated skeletal muscles in chick embryo. Proc. Natl. Acad. Sci. U. S. A. *70:* 1708–1712.

Gurney, M. D. (1984) Suppression of terminal sprouting at the neuromuscular junction by immune sera. Nature *307:* 546–548.

Hoffman, H. (1950) Local reinnervation in partially denervated muscle. A histophysiological study. Aust. J. Exp. Biol. Med. Sci. *28:* 383–397.

Hoffman, H., and P. H. Springell (1951) An attempt at the chemical identification of neurocletin (the substance evoking axon sprouts). Aust. J. Exp. Biol. Med. Sci. *29:* 417–424.

Holland, R. L., and M. C. Brown (1980) Postsynaptic transmission block can cause terminal sprouting of a motor nerve. Science *207:* 649–651.

Huang, C. L-H., and R. J. Keynes (1983) Terminal sprouting of mouse motor nerves when the post-synaptic membrane degenerates. Brain Res. *274:* 225–229.

Kemplay, S., and C. Stolkin (1980) Endplate classification and spontaneous sprouting in the sternocostalis muscle of the rat: A new whole mount preparation. Cell Tissue Res. *212:* 333–339.

Lavoie, P. A., G. Collier, and A. Tenenhouse (1976) Comparison of α-bungarotoxin binding to skeletal muscles after inactivity or denervation. Nature *260:* 349–350.

Lee, C. Y. (1972) Chemistry and pharmacology of polypeptide toxins in snake venoms. Annu. Rev. Pharmacol. *12:* 265–286.

Marshall, L. M., J. R. Sanes, and U. J. McMahan (1977) Reinnervation of original synaptic sites on muscle fiber basement membrane after disruption of the muscle cells. Proc. Natl. Acad. Sci. U. S. A. *74:* 3073–3077.

Mathers, D. A., and S. Thesleff (1978) Studies on neurotrophic regulation of murine skeletal muscle. J. Physiol. (Lond). *282:* 105–114.

Miledi, R., and L. T. Potter (1971) Acetylcholine receptors in muscle fibers. Nature *233:* 599–603.

O'Brien, R., A. Ostberg, and G. Vrbova (1978) Observations on the elimination of polyneuronal innervation in developing muscle. J. Physiol. (Lond.) *282:* 571–582.

Pestronk, A., and D. B. Drachman (1976) Effect of botulinum on trophic regulation of acetylcholine receptors. Nature *264:* 787–789.

Pestronk, A., and D. B. Drachman (1978a) A new stain for quantitative measurement of sprouting at neuromuscular junctions. Muscle Nerve *1:* 70–74.

Pestronk, A., and D. B. Drachman (1978b) Motor nerve sprouting and acetylcholine receptors. Science *199:* 1223–1225.

Pestronk, A., D. B. Drachman, and J. W. Griffin (1976) Effect of muscle disuse on acetylcholine receptors. Nature *260:* 352–353.

Pestronk, A., D. B. Drachman, and J. W. Griffin (1980) Effects of aging on sprouting and regeneration. Exp. Neurol. *70:* 62–85.

Pestronk, A., D. B. Drachman, R. Teoh, and R. N. Adams (1983) Combined short-term immunotherapy for experimental autoimmune mysthenia gravis. Ann. Neurol. *14:* 235–241.

Ramón y Cajal, S. (1928) *Degeneration and Regeneration of the Nervous System*, Vol. 1, by S. May, transl., Oxford University Press. Cambridge England.

Ruffolo, R. R., Jr., G. S. Eisenbarth, J. M. Thompson, and M. Nirenberg (1978) Synapse turnover: A mechanism for acquiring synapse specificity. Proc. Natl. Acad. Sci. U. S. A. *75:* 2281–2285.

Smith, D. O., and J. L. Rosenheimer (1982) Decreased sprouting and degeneration of nerve terminals of active muscles in aged rats. J. Neurophysiol. *48:* 100–109.

Tonge, D. A. (1974) Chronic effects of botulinum toxin on neuromuscular transmission and sensitivity to acetylcholine in slow and fast skeletal muscle of the mouse. J. Physiol. (Lond.) *241:* 127–139.

Toyka, K. V., K. L. Birnberger, A. P. Anzil, C. Schlegel, U. Besinger, and A. Struppler (1978) Myasthenia gravis: Further electrophysiological and ultrastructural analysis of transmission failure in the mouse passive transfer model. J. Neurol. Neurosurg. Psychiatry *41:* 746–753.

Tuffery, A. R. (1971) Growth and degeneration of motor endplates in normal cat hind limb muscles. J. Anat. *110:* 221–247.

Wernig, A., M. Pecot-Dechavassine, and H. Stover (1980) Sprouting and regression of the nerve at frog neuromuscular junction in normal conditions and after prolonged paralysis with curare. J. Neurocytol. *9:* 277–303.