Motor Nerve Sprouting and Acetylcholine Receptors

Abstract. *Sprouting of motor nerve terminals was evoked by functional denervation of skeletal muscles brought about by presynaptic blockade or disuse. The amount of sprouting, determined by morphometric measurement, was correlated with the level of extrajunctional acetylcholine receptors. Sprouting was inhibited by blockade of acetylcholine receptors with α-bungarotoxin. Extrajunctional acetylcholine receptors may play an important role in eliciting motor nerve terminal sprouting.*

Motor nerves retain the ability to sprout throughout adult life (*1*). The sprouting process is thought to play an important role in the reinnervation of denervated muscle (*2*), as well as in the maintenance of normal nerve terminals (*3*). There is evidence that sprouting occurs in a variety of clinical and experimental situations in which muscle undergoes denervation changes, including motor neuron disease (*4*), myasthenia gravis (*5*), botulinum toxin poisoning (*6*), and muscle disuse (*7*). However, the factors that elicit the sprouting response are not yet understood.

To study the relationship between muscle denervation and motor nerve sprouting we produced denervation changes in muscle by three different means and measured their effects on nerve terminal sprouting by a morphometric method. Functional denervation of muscle was brought about by (i) presynaptic blockade of acetylcholine (ACh) transmission, produced by botulinum toxin (*8, 9*); (ii) muscle disuse, due to tetrodotoxin (TTX) blockade of nerve impulse conduction (*10, 11*); and (iii) postsynaptic blockade of neuromuscular transmission, produced by α-bungarotoxin (α-BuTx) (*12*). As an indicator of denervation changes produced in the muscles by each of these procedures, we measured the extrajunctional ACh receptors by means of [^{125}I]-α-BuTx binding. To evaluate nerve sprouting quantitatively, we developed a histological-histochemical method for staining and measuring neuromuscular junctions. The results suggest a close relationship between the degree of motor nerve sprouting and the level of available extrajunctional ACh receptors. Experiments were performed with female Sprague-Dawley rats (200 to 225 g) under chloral hydrate anesthesia (400 mg/kg).

Presynaptic blockade was produced by botulinum toxin, a purified protein that has been shown to block quantal release of ACh at nerve terminals in a highly specific manner (*13*). Neuromuscular blockade lasts for several weeks after a single dose of the toxin (*8, 9*). Crystalline type A botulinum toxin (*14*) was freshly diluted in Ringer solution before use. A volume of 30 μl containing 1.2×10^{-9} g was injected into the soleus muscle through a fine No. 30 needle. Seven days later, neuromuscular blockade was verified by nerve stimulation and direct observation of the isolated muscle, as previously described (*9*). Only muscles that were completely paralyzed were used for further studies. Control animals received single intramuscular injections of 10 μg of bovine serum albumin in 30 μl of Ringer solution on day zero.

Muscle disuse was maintained by repeated microinjection of TTX into the sciatic nerve. We have previously shown that the TTX method eliminates all nerve-mediated muscle usage without damaging the nerves structurally or interfering with spontaneous ACh release or axonal transport (*10*). Subperineurial injections of 2 μg of TTX in 2 μl of Ringer solution were made with glass micropipettes at 48-hour intervals. Control animals were given injections of Ringer solution alone. Before each repeat injection of TTX, and at the termination of each experiment, impulse blockade was tested by nerve stimulation. Only animals with complete blockade throughout the 7-day experimental period were retained.

Postsynaptic blockade was brought about by the use of α-BuTx, a purified fraction of the venom of the snake *Bungarus multicinctus* that binds specifically and essentially irreversibly to ACh receptors (*15*). Injections of α-BuTx, 3 μg in 30 μl of Ringer solution, were made directly into the soleus muscle every 24 hours from day 0 to day 5. This procedure blocked more than 90 percent of the junctional ACh receptors throughout the 24-hour interval between injections (*16*), although the muscles were not completely paralyzed at the end of each daily period, as also noted by others (*17, 18*). On day 6 of the experiment, injections of purified *Naja naja siamensis* α-toxin (3 μg in 30 μl of Ringer solution) were substituted for α-BuTx. This toxin, which also specifically blocks ACh receptors (*19*), was used for the final injection instead of α-BuTx because the reversibility of its effect permits subsequent determination of the number of extrajunctional ACh receptors by [^{125}I]-α-BuTx binding (*20*). Control animals received intramuscular injections of 10 μg of albumin in 30 μl of Ringer solution on days 0 to 6 directly into the soleus muscle.

Seven days after the beginning of each experimental procedure, the rats were anesthetized, and each soleus muscle was removed and teased longitudinally into two pieces. The larger piece was used for histology. It was pinned at resting length and quickly frozen in isopentane cooled with solid CO_2. Longitudinal sections, 50 μm thick, were cut in a cryostat, placed on glass slides in a drop of 3 percent EDTA, and allowed to dry. Cholinesterase staining was performed by a bromoindoxylacetate method. Nerve terminals were demonstrated by fixing in neutral Formol saline, pretreating slides in a solution of 1.5 percent pyridine (by volume) and 10 percent chloral hydrate (weight to volume), staining with silver nitrate, developing in a hydroquinone and sodium sulfite solution, and toning with sodium tetrachloroaurate. Full details of the staining procedure have been presented elsewhere (*21*). This method displays the cholinesterase-containing end plate as a well-demarcated transparent blue zone, against which the black silver-stained nerve terminals stand out clearly.

The degree of sprouting was evaluated quantitatively, as illustrated in Fig. 1, by (i) counting the number of nerve terminal

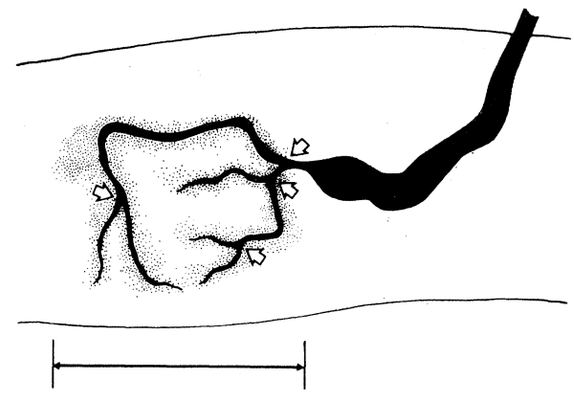

Fig. 1. Diagram showing morphometry of neuromuscular junction. The end plate length, outlined by the cholinesterase stain (dotted area), is measured parallel to the length of the muscle fiber (interval marker). The nerve terminal branch points are indicated by the open arrows.

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branch points within each end plate area, and (ii) measuring the length of each end plate area, as outlined by the cholinesterase stain, parallel to the length of the muscle fiber. The sprouting index is the mean number of branch points for each muscle multiplied by the mean end plate length (micrometers) for the same muscle and is useful for expressing the degree of terminal sprouting as a single number.

The second piece of soleus muscle was used for determinations of extrajunctional ACh receptors by an [^{125}I]-α-BuTx binding method (*10*). The specific and irreversible binding of this snake venom fraction to ACh receptors has been used extensively for quantitative measurements of extrajunctional ACh receptors (*15*). This determination is possible even in the animals previously treated with α-BuTx. The rapid turnover of extrajunctional ACh receptors (*17*) results in replacement of virtually all α-BuTx–blocked extrajunctional receptors by newly incorporated receptors within 48 hours, permitting subsequent quantitation with [^{125}I]-α-BuTx (*22*).

There were two main findings. First, there was a close correlation between the degree of sprouting and the measured levels of extrajunctional receptors, except in the postsynaptic blockade experiments (Table 1). Second, in experiments where α-BuTx was used to produce postsynaptic blockade, there was no significant sprouting as compared with the controls (Table 1).

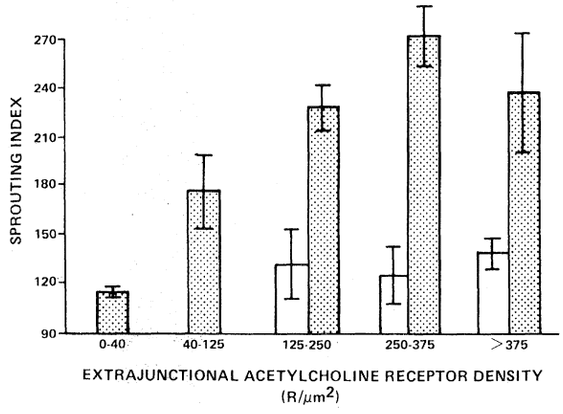

Fig. 2. Relation of sprouting index to extrajunctional ACh receptor density. Stippled columns represent data from all muscles treated with botulinum toxin, TTX disuse, and various control procedures. Open columns represent data from all muscles treated with α-BuTx or α-BuTx plus botulinum toxin. Note the marked inhibition of sprouting produced by α-BuTx at all levels of extrajunctional ACh receptors. Each column represents the mean ± standard error for 4 to 13 muscles.

Table 1. Effects of various treatments on sprouting and extrajunctional ACh receptors. Each experimental procedure is grouped with its appropriate treatment control. Details of the individual treatment controls are given in the text. Numbers in parentheses are numbers of muscles sampled. At least 20 end plates were measured in each muscle.

Treatment	Terminal branch points	End plate length (μm)	Sprouting index	Extrajunctional ACh receptor density (No./μm^2)
Untreated control	2.6 ± 0.1 (7)	38 ± 2	101 ± 6	22 ± 2
Botulinum	4.7 ± 0.1 (7)*	60 ± 3*	285 ± 18*	308 ± 20*
Control for botulinum	3.0 ± 0.1 (11)	38 ± 1	112 ± 5	22 ± 3
Tetrodotoxin, intraneural	4.1 ± 0.2 (6)*	49 ± 2*	206 ± 13*	106 ± 19*
Control for tetrodotoxin	2.8 ± 0.2 (3)	35 ± 1	98 ± 8	22 ± 2
α-Bungarotoxin	3.4 ± 0.2 (6)	40 ± 2	138 ± 18	251 ± 55*
Control for α-bungarotoxin	3.5 ± 0.1 (6)†	38 ± 2	133 ± 9†	38 ± 7†

*Significantly different from experimental control, $P < .001$. †Significantly different from untreated control, $P < .01$.

Table 2. Effect of α-bungarotoxin and control procedures on botulinum-induced sprouting. Results are expressed as means ± standard errors. Numbers in parentheses indicate muscles sampled. At least 20 end plates were measured in each muscle.

Treatment	Terminal branch points	End plate length (μm)	Sprouting index	Extrajunctional ACh receptor density (No./μm^2)
Botulinum and albumin	4.7 ± 0.2 (8)	49 ± 2	230 ± 16	275 ± 46
Botulinum and α-bungarotoxin	3.2 ± 0.2 (8)*,†	40 ± 1*,†	129 ± 10*,†	338 ± 40
Ringer and albumin	3.5 ± 0.1 (6)*	38 ± 2*	133 ± 9*	38 ± 7*

*Significantly different from botulinum control, $P < .01$. †Not different from experimental control, $P > .1$

Our results (Table 1 and Fig. 2) show a correlation between sprouting and ACh receptors, reaching a maximum sprouting index at a receptor density of about 350 receptors per square micrometer ($r = .9$, $P << .001$). More data will be needed to establish the shape of the curve at high ACh receptor levels.

In contrast to these results, no significant sprouting occurred after injections of α-BuTx, in spite of the development of high levels of extrajunctional ACh receptors (Table 1). Nerve terminal branching and end plate length in α-BuTx–treated muscles were not different from those in the control muscles ($P > .1$).

There are at least two possible explanations for the failure of α-BuTx–induced postsynaptic blockade to elicit sprouting: (i) the resulting denervation changes in muscle may not be sufficient or appropriate to evoke nerve terminal sprouting, and (ii) α-BuTx itself might interfere with sprouting.

To resolve this problem, we tested the ability of α-BuTx to inhibit sprouting by applying it in a situation where a pronounced sprouting response otherwise occurs—that is, after botulinum intoxication. In this experiment, 16 rats were first given injections of botulinum toxin into the soleus muscle, as before. In addition, eight of these animals received

daily intramuscular injections of 3 μg of α-BuTx; the other eight received control injections of albumin-Ringer solution. The results showed virtually complete inhibition of sprouting in the muscles injected with α-BuTx in addition to botulinum toxin, while the other muscles exhibited a marked sprouting response (Table 2). Both procedures resulted in greatly elevated levels of extrajunctional ACh receptors. Thus, α-BuTx appears to inhibit the sprouting response without reducing the denervation changes in muscle (Fig. 2).

That this inhibitory effect of α-BuTx was not due to interference with the nerve's ability to sprout was shown by a further set of experiments. Axonal outgrowth was initiated by crushing, a procedure that directly stimulates sprouting from the proximal nerve stump. The nerve to the soleus was crushed with jewelers forceps in 12 rats at the point of entry into the muscle. One group of eight rats received six daily intramuscular injections of α-BuTx, while the control group were given injections of albumin-Ringer solution. Histological preparations after 7 days revealed abundant outgrowth of axons from the crushed nerves in both the α-BuTx–treated and control groups. This finding excludes the possibility of a general sprout-inhibiting effect of α-BuTx.

It thus seems most likely that the inhibition of nerve terminal sprouting produced by α-BuTx results from its only known action, highly specific and irreversible blockade of ACh receptors. The mechanism by which receptor blockade might inhibit sprouting is not yet certain. However, the results of our experiments and other recent reports (*7, 24*) provide suggestive evidence that the extrajunctional ACh receptors, which increase in denervated muscle, may play a role in eliciting motor nerve terminal sprouting. First, functional denervation produced by botulinum toxin or by disuse caused by TTX resulted in terminal sprouting. The experimental conditions excluded the possibility that incidental nerve or muscle damage might have been instrumental in eliciting the sprouting response. More important, the degree of sprouting was closely correlated with the level of extrajunctional ACh receptors. Second, procedures that reduce or block extrajunctional ACh receptors appear to inhibit the tendency to sprout. In one study, electrical stimulation of botulinum-treated muscle decreased both extrajunctional receptors and nerve sprouting (*24*). We now report that a specific pharmacological blocker of ACh receptors also prevents sprouting. Whether it does so by blocking the ACh recognition site itself, by sterically hindering a nearby site, or by some as yet undescribed action of α-BuTx remains a matter for future study. Although these findings do not exclude the participation of additional factors in eliciting sprouting at motor nerve terminals, they suggest an important role for ACh receptors.

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22. To verify this, we measured extrajunctional ACh receptors in two groups of 7-day denervated soleus muscles: one group was injected with α-BuTx 48 hours before the end of the experiment, while the other served as controls. There was no difference in receptor levels between the two groups ($P > .1$). Moreover, the final injection of *Naja naja* toxin on day 6 does not interfere with the receptor assay, since the binding of this toxin to ACh receptors is reversible, and it is displaced on incubation with [^{125}I]-α-BuTx (*20*).
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Merging of Modalities in the Optic Tectum: Infrared and Visual Integration in Rattlesnakes

Abstract. *The optic tectum of pit vipers (Crotalinae) contains a layer of infrared-sensitive neurons subjacent to the visual layer; these indirectly receive input from the facial pit organs. They respond transiently to the appearance or motion of warm objects within their 25° to 70° excitatory receptive fields (some have inhibitory regions) and presumably allow the snake to orient or strike toward prey. The infrared and visual spatiotopic tectal maps have similar but not identical axes; the infrared magnification is greater than that for vision. Bimodal neurons have receptive fields for each modality that reflect the disparity of the two maps. This finding suggests that (i) during development the infrared and visual fibers spread out independently to fill available tectal sites and (ii) bimodal neurons form local connections without regard to establishing spatial correspondence between the two modalities.*

The superior colliculus, or optic tectum, is widely recognized as a center for visual integration with primary roles in the control of eye movements (*1, 2*) and the orientation of the head or body toward a stimulus source, such as for prey capture (*2, 3*). Other sensory modalities are also represented by the responses of tectal neurons: auditory, somatic, proprioceptive, and multimodal responses are recorded in cells of the mammalian superior colliculus (*4–6*). Tectal circuitry may therefore be hypothesized to bring sources of visual and nonvisual stimulation to the same central region of the animal's visual field or zone of action. In this report, we explore the question of how similarly space is represented in the optic tectum in two distinct modalities; spatiotopic maps and the receptive fields of bimodal neurons suggest that similar principles determine the connections of each modality but that no mechanism operates to ensure that one region of space is represented at the same tectal locus or by the same tectal neurons.

In rattlesnakes, (fam. Viperidae, sub fam. Crotalinae), the facial pits, specialized bilateral infrared sense organs, detect the appearance and motion of dis-

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