less) and a continuous pattern of firing.

In conclusion, we believe that this in vitro technique may give valuable information on neuronal properties of NS cells and provide model systems for studying the nature of the synthetic, transport, release, and uptake processes associated with the NS phenomenon.

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Neuromuscular Junction in Myasthenia Gravis: Decreased Acetylcholine Receptors

Abstract. *The number of acetylcholine receptors was determined in the neuromuscular junctions of eight patients with typical myasthenia gravis and in five controls, by means of ^{125}I-labeled α-bungarotoxin binding. The junctional acetylcholine receptors were reduced in the myasthenic muscles as compared with the controls. This reduction in receptors may account for the defect in neuromuscular transmission in myasthenia gravis.*

Myasthenia gravis is a neuromuscular disorder manifested by muscular weakness and fatigability. Although the abnormality is thought to involve the neuromuscular junction, the precise nature of the defect is still uncertain. There has been considerable debate as to whether the nerve terminal or the postsynaptic region of muscle, or both, are affected by the disease process (*1*). We have examined the number and distribution of acetylcholine (ACh) receptors in a series of patients with myasthenia gravis and in a control group of patients.

Eight consecutive patients with typical myasthenia gravis, all of whom gave informed consent, were included in the study. In each case, the diagnosis was established by a typical history and physical findings of muscular weakness and fatigue. The diagnosis was confirmed by a decline of more than 15 percent in the amplitude of the third or fourth muscle action potential in response to a train of supramaximal stimuli applied at a rate of two to five per second (*2*). All eight patients showed improvement in muscle strength after injection of edrophonium hydrochloride (Tensilon) and after oral administration of pyridostigmine bromide (Mestinon). Six of the eight patients had been treated with anticholinesterase medication for a year or more, while the other two were not under treatment at the time of biopsy. One additional patient who manifested typical symptoms of myasthenia gravis 2 years earlier, but whose status had changed in the interim, was also included. Although still weak, he no longer had the clinical fatigability or the electrodiagnostic and pharmacological characteristics of myasthenia gravis. The control group consisted of a series of patients with a variety of problems ranging from aching pains to various neuromuscular disorders. Two of the control patients, H.H. and D.L., were considered normal.

Motor point biopsies were obtained by a modification of the method of Coers and Woolf (*3*). Anticholinesterase medication was withheld in all cases for a minimum of 10 hours prior to the biopsy, and when necessary, constant nursing care was given during this period. At open biopsy, the "motor point" was identified as the locus at which a visible twitch response could

Table 1. Acetylcholine receptor sites in control and myasthenic patients; S.E.M., standard error of the mean.

Patient			Diagnosis	Medication*	Tensilon response	Change in evoked potentials (%)	Receptor sites per junction† (Mean ± S.E.M.)	Relative grain density‡
Name	Age	Sex						
D.L.	50	M	Cerebral ischemia	Yes			$4.09 \times 10^7 \pm 0.50$	3.9
J.C.	19	M	Scapuloperoneal dystrophy	No			$3.63 \times 10^7 \pm 0.27$	
H.H.	55	M	Muscle aching	No			$5.72 \times 10^7 \pm 0.51$	3.6
J.R.	57	M	Lambert-Eaton syndrome	Yes		+ 200	$3.45 \times 10^7 \pm 0.35$	
J.A.R.	18	F	Facioscapulohumeral dystrophy	No			$2.19 \times 10^7 \pm 0.21$	
A.N.	38	F	Myasthenia gravis	None for 4 months	+	− 37	$1.15 \times 10^7 \pm 0.29$	1.0
T.T.	20	F	Myasthenia gravis	Yes	+	− 33	$0.41 \times 10^7 \pm 0.10$	1.4
T.H.	63	M	Myasthenia gravis	Yes	+	− 50	$0.54 \times 10^7 \pm 0.08$	1.2
V.F.	60	F	Myasthenia gravis	Yes	+	− 21	$0.68 \times 10^7 \pm 0.23$	
A.F.	54	F	Myasthenia gravis	Yes	+	− 15		1.5
F.S.	29	F	Myasthenia gravis	Yes	+	− 50		1.9
M.M.	41	F	Myasthenia gravis	No	+	− 30		1.8
E.D.	26	F	Myasthenia gravis	Yes	+	− 30		2.0
P.B.	76	M	Myasthenia gravis, in remission	Yes	−§ +¶	0‖ − 50	$3.56 \times 10^7 \pm 0.21$	

* Cholinesterase inhibitors were given as described in the text. † Number of α-bungarotoxin-binding sites determined with ^{125}I-labeled α-bungarotoxin. ‡ At neuromuscular junction from autoradiograms. The method of grading is described in the text. § April 1973. ‖ March 1973. ¶ June 1972.

be elicited by a minimal electrical stimulus applied directly to the surface of the muscle with a sterile silver electrode. Several bundles of muscle fibers were removed, stretched to approximately resting length, and pinned in dishes in a nutrient medium (*4*). Small groups of fibers were dissected from the bundles, separately pinned, and incubated at 37°C for 2 to 2½ hours in fresh medium containing 0.2 to 0.4 μg of ^{125}I-labeled α-bungarotoxin per milliliter (*5*). α-Bungarotoxin interacts specifically and in an essentially irreversible manner with ACh receptors and thus has been used to determine the number of ACh receptor sites (*6, 7*). The number of α-bungarotoxin-binding sites is proportional (perhaps equal) to the number of ACh-receptor sites, and the terms are therefore used interchangeably in this report.

For each biopsy, autoradiographic or scintillation counting techniques (*7*), or both, were used. The toxin-treated, washed, and glutaraldehyde-fixed fibers were stained for acetylcholinesterase (AChE) (*8*) to reveal neuromuscular junctions. For autoradiographic analysis, lengths of single muscle fibers including the neuromuscular junction were dissected out, mounted on microscope slides, and processed for autoradiography. For quantitative analysis of junctional ACh receptors, short segments of muscle bundles containing up to 50 neuromuscular junctions were dissected out and placed on microscope slides for counting the number of junctions in each group. Groups of fibers without junctions were handled similarly for determination of extrajunctional binding sites (*9*). Each group of fibers was hydrolyzed in 100 μl of 6*N* HCl at 115°C for 48 to 72 hours in a sealed glass tube. The tubes were then cut open and inverted in scintillation vials to which were added 0.9 ml of 1 percent Triton X-100 in water and 10 ml of Triton-toluene fluor (*10*). Samples were counted for at least 40 minutes each in a Packard scintillation spectrometer (60 percent efficiency).

Table 2. Effect of neostigmine treatment on ACh receptor sites in rats; *N*, number of observations; S.E.M., standard error of the mean.

Treatment	*N*	Diaphragms (No.)	Receptor sites per junction (mean ± S.E.M.)
None	130	5	$2.81 \times 10^7 \pm 0.07$
< 24 hours*	26	3	$3.27 \times 10^7 \pm 0.17$
14 days†	22	2	$1.48 \times 10^7 \pm 0.05$
34 days†	32	4	$1.83 \times 10^7 \pm 0.10$

* A total of 50 to 75 μg was injected subcutaneously. One or two injections were given to each animal. † The dosage was 100 μg injected subcutaneously twice daily. For the first 5 days, 50- to 75-μg doses were given, to avoid excessive mortality. The rats (females) weighed from 180 to 225 g at death.

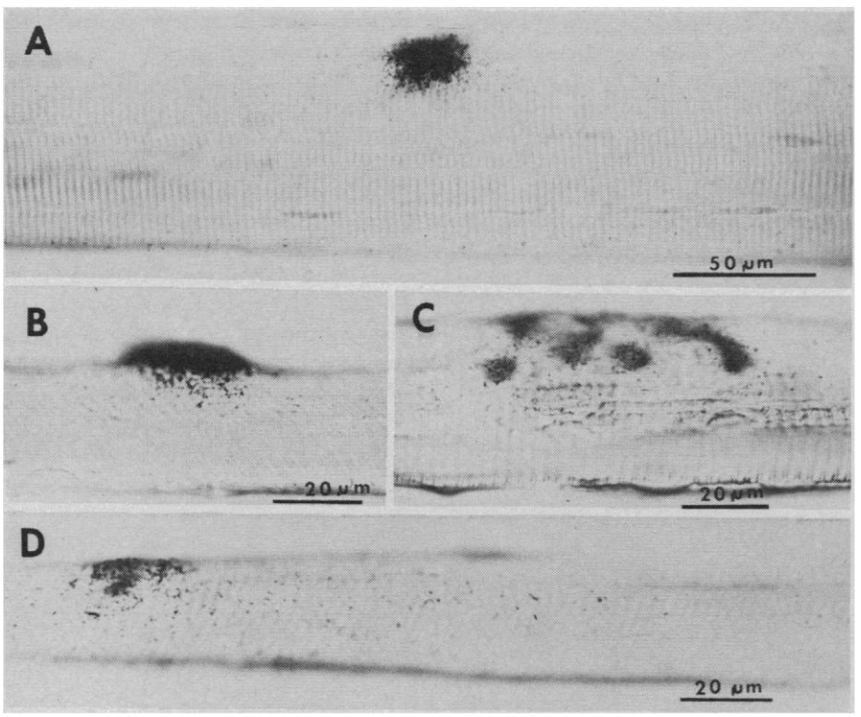

Fig. 1. Autoradiograms of human muscle fibers after incubation in ^{125}I-labeled α-bungarotoxin and staining for acetylcholinesterase to reveal neuromuscular junctions. (A and B) Normal fibers from two patients (H.H. and N.A.). There is a dense accumulation of silver grains over a limited junctional area and a paucity of grains outside this region. (C) Myasthenic fiber (patient A.N.). The number of grains is markedly reduced, but grains are mostly localized over cholinesterase-stained area. (D) Myasthenic fibers (patient F.S.) showing decreased grain density at neuromuscular junction and a relatively high grain density over the adjacent extrajunctional region. Specific activities of the ^{125}I-labeled α-bungarotoxin were 2.2×10^4 to 3.4×10^4 c/mole, and exposure times were 4 days for (A) and (D), and 5 days for (B) and (C). On the relative grain density scale described in the text, (A) and (B) are ranked 4, (C) is ranked 1, and (D) is ranked 2.

In the autoradiographs of myasthenic fibers, the number of grains over neuromuscular junctions was greatly reduced (Fig. 1). In order to evaluate them without bias, two of us (D.F. and D.B.D.) studied all the autoradiographic slides after they had been coded in random order, and graded the grain density over the junctions as follows: 4, normal; 3, possibly reduced; 2, definitely reduced; and 1, markedly reduced. Our estimates were in close agreement; the grade indicated in Table 1 is the mean of the grades given to all the slides by both observers. In three fiber samples (F.S., M.M., and A.N.), the autoradiograms showed grains extending beyond the ChE-stained junction (Fig. 1D).

The results of scintillation counting are summarized in Table 1. In the control group, the mean number of receptors per neuromuscular junction was 3.81×10^7. By contrast, the neuromuscular junctions of the patients with typical myasthenia gravis contained approximately 11 to 30 percent of this number of ACh receptors. The values in the myasthenic and control groups were significantly different ($P < .01$) (*11*). The junctions of patient P.B., the above-described former myasthenic, contained a normal number of receptor sites. The number of extrajunctional receptor sites in the four myasthenic muscles analyzed by scintillation counting was not above those of the five control patients.

Of the eight myasthenic patients, six were being treated with the anticholinesterase drug pyridostigmine bromide. One patient (A.N.) had received no anticholinesterase medication for more than 4 months and one (M.M.) had never had such medication. Since the findings in these last two patients did not differ from the others in ACh receptor number or distribution, and since two of the "control" patients who had taken anticholinesterase medica-

tion for more than a year had a normal receptor number (D.L. and J.R.), our results cannot be attributed solely to an effect of medication. Nevertheless, to obtain some idea of the effects of anticholinesterase treatment on α-bungarotoxin-binding receptor sites, we studied the effects of the AChE inhibitor neostigmine bromide in rats (Table 2). Short-term administration (less than 24 hours) of neostigmine had no effect upon measured receptor number. Administration for 14 to 34 days led to a decrease in junctional receptor number which was, however, not as great as that seen in the myasthenic junctions. Similar results in rats have just been reported (*12*). However, the dosage given to the rats in both studies was 10 to 20 times the recommended parenteral doses in man (*13*), on a weight basis. In view of the differences in species and dosages, we cannot be certain whether long-term treatment with cholinesterase inhibitors can lead, in humans, to reduced numbers of ACh receptors, although it did not in our two control group cases. Since a reduction in ACh receptors would be highly undesirable for myasthenic patients, this matter warrants further investigation.

Our study shows a deficit in junctional ACh receptors in myasthenia gravis. This deficit might result from a major decrease in postsynaptic surface area. However, information now available does not support a decrease sufficient to account for the observed reduction in ACh receptor number. Light (*14*) and electron microscope (*15*) studies have shown that myasthenic junctions have less highly folded postsynaptic surfaces than normal and are excessively elongated (*16*). However, no attempts have been made to measure total postsynaptic surface area. It seems more likely that the deficit in ACh receptor number is due to a reduction in the number of functional ACh receptors in myasthenic junctions without a proportionate decrease in postsynaptic area. Such a reduction might reflect a decreased packing density of receptor molecules in postsynaptic membranes or some molecular alteration (*17*) or endogenous blockade of a large proportion of the normal complement of ACh receptors.

A reduction in the number of ACh receptors at myasthenic neuromuscular junctions may well be a consequence of some other lesion. Moreover, we cannot exclude the possibility that some patients with the clinical syndrome of myasthenia gravis may have a different neuromuscular defect from that reported here. However, a reduction in the number of functional ACh receptors in the postsynaptic membrane, as indicated by our data, would contribute to the physiological abnormalities (*18*) of myasthenia gravis.

Note added in proof: In order to directly test the adequacy of our incubation conditions (*5*), the left deltoid muscle of an additional myasthenic patient was biopsied. This patient, K.W., is a 60-year-old female with a positive response to Tensilon and a greater than 20 percent decrease in evoked potentials. The number of α-bungarotoxin binding sites per neuromuscular junction was measured after incubations of 1, 2, and 3 hours with 0.36 μg of toxin per milliliter. These samples yielded values of 0.85×10^7, 0.82×10^7, and 0.74×10^7 sites per neuromuscular junction, respectively.

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4. A modified Ham's F-12 medium, containing 0.5 percent bovine serum albumin, in which the sodium salt of Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate) (*p*H 7.2; 18 m*M*) replaced $NaHCO_3$.
5. The groups of muscle fibers were about 1 mm in diameter and usually 3 to 5 cm long. The incubation conditions were chosen to be more than sufficient to saturate specific α-bungarotoxin-binding sites in rat diaphragm. Some unique permeability barrier in myasthenic muscle might render these conditions insufficient. We know of no ultrastructural evidence that suggests such a barrier. We would expect binding to superficial fibers to saturate in less than 1 hour in any case, and these should appear normal in autoradiograms. Such fibers were not observed. Nevertheless, we cannot rigorously exclude this possible source of error. After incubation in medium containing bungarotoxin, the muscle was washed 12 times for 5 minutes each time in cold (2° to 10°C), Hepes-buffered balanced salt solution containing 0.5 percent bovine serum albumin, then stored overnight at 2°C in washing medium, and rinsed four more times with cold medium before glutaraldehyde fixation.
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9. Groups of fibers containing or lacking neuromuscular junctions were cut to approximately 2 mm lengths. Extrajunctional binding sites were determined on a per fiber-length basis. Such binding sites usually represented 1 to 3 percent of the junctional binding sites for controls, which corresponds to approximately five to ten binding sites per square micrometer of muscle surface. Myasthenics and controls were not significantly different in this respect. In Table 1 correction has been made for extrajunctional binding.
10. Each liter contained 2.8 g of PPO (2,5-diphenyloxazole), 0.035 g of POPOP (1,4-bis[2-(5-phenyloxazolyl)benzene]). 665 ml of toluene, and 333 ml of Triton X-100.
11. For each biopsy 5 to 13 separate samples containing junctions were counted. The original scale (bungarotoxin sites per junction) was transformed to $(10^3 \cdot \text{bungarotoxin sites/junction})^{1/2}$. Variance was analyzed on the transformed scale to estimate variance components. Confidence intervals were determined by back-transforming to the original scale. Professor Alan Ross performed the statistical analysis.
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16. The mean length of myasthenic junctions in our series was 66.5 μm ($N = 285$), measured in AChE-stained preparations, as compared with 36.0 μm for the controls ($N = 188$). The controls included one patient, J.C., with elongated junctions. Excluding J.C., the control value is 27.7 μm ($N = 172$). The mean length is somewhat misleading since it includes nonjunctional regions interposed between the multiple esterase-reactive regions. By light microscopy, the esterase-reactive regions often appear to be substantially increased in area as well, but we know of no direct measurements of this parameter. In almost all cases the number of endplates was identical to the number of fibers in each hydrolysis sample. Multiple innervation—that is, two or more well-separated esterase-reactive areas—was rare except in patient P.B. In this case each separate area was considered a neuromuscular junction. On a per fiber basis his junctional α-bungarotoxin-binding sites would be about 8 percent greater or 3.84×10^7 sites per fiber.
17. An alteration in the postsynaptic membrane which drastically decreased the rate of association of bungarotoxin with receptor or which rendered this association readily reversible would not be distinguished from a reduced number of receptors in the experiments reported here.
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