known functional requirements that have permitted this degree of variability during evolution.

Since the four known subclasses of γ chains are under the control of a series of closely linked genes (*18*), it seems particularly striking that, as is shown in Fig. 3, γHCD proteins belonging to three of these subclasses have normal sequences after the same position. Thus it seems possible that Glu at residue 216 near the hinge region could represent the beginning of another gene. The data on two other immunoglobulins with large deletions do not provide further support for this possibility. The deletion in protein Hi has not been defined sufficiently to be informative (*7, 19*). Protein Sac, an unusual γG1 myeloma protein, has a light chain with an internal deletion of about 70 residues, and a heavy chain lacking the first 102 residues (*20*). The possibility that the findings in the heavy chain are the result of proteolysis has not been excluded. Smithies has proposed a branched-chain DNA for immunoglobulin genes, one that would result in varying deletions dependent on DNA breakage and differing mechanisms of repair (*21*). This could explain the results of the three HCD proteins if codon 216 was particularly susceptible to DNA breakage. The limited number of proteins studied makes any proposed mechanism highly speculative, but future studies may establish the reason for the unusual structure of the hinge region and provide the mechanism by which two, three, or more genes are integrated so that a single polypeptide chain is synthesized.

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5. Numbering is based on the sequence of γ1 proteins because no γ2 myeloma has been completely sequenced. Protein Eu was described by G. M. Edelman, B. A. Cunningham, W. E. Gall, P. D. Gottlieb, U. Rutishauser, M. J. Waxdal [*Proc. Nat. Acad. Sci. U.S.* **63**, 78 (1969)]; protein DAW was described by Press and Hogg (*13*).
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Acetylcholine Receptors: Number and Distribution at Neuromuscular Junctions in Rat Diaphragm

Abstract. *The number of acetylcholine receptors per motor end plate in the rat diaphragm, measured by the binding of [^{125}I]α-bungarotoxin, varies directly with rat size and is (4.0 ± 0.2) × 10^7 for full-grown male rats. Autoradiographic analysis of single fibers labeled with this substance reveals that virtually all of these receptors are localized in the end plate.*

α-Bungarotoxin (α-BGT), a polypeptide isolated from the venom of *Bungarus multicinctus*, binds tightly to skeletal muscle and electroplax membranes in the microregion of the acetylcholine receptors and blocks the response of these cells to acetylcholine (ACh) (*1–3*). The polypeptide binds specifically to ACh-sensitive cells (*4*), and both ACh and *d*-tubocurarine compete with α-BGT in the binding reaction (*1, 2*). For these reasons, α-BGT labeled with ^{125}I or ^{131}I is used as a tag for the ACh receptor. We have used [^{125}I]α-BGT to determine the number and spatial distribution of ACh receptors in the muscle membranes at rat neuromuscular junctions.

To determine the number of receptors per end plate, we pinned the left hemidiaphragms of male and female Sprague-Dawley rats (100 to 380 g) to stainless steel grids; in each hemidiaphragm, the endplate region of a layer of deep muscle fibers was exposed by dissecting along the phrenic nerve as it courses through the muscle mass. The pinned diaphragms were incubated for 2 hours at 37°C in modified Ham's F12 culture medium, which was buffered with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid as zwitterion and contained [^{125}I]α-BGT, 10^{-6} g/ml. Then the diaphragms were transferred to fresh medium that lacked α-BGT, washed with 12 changes of medium for 5 minutes each, fixed in glutaraldehyde, and stained for the end-plate marker acetylcholinesterase (AChE) (*5*). Groups of muscle fiber segments containing 0 to 60 end plates were dissected from the muscle, the number of end plates in each group was determined, and each group was hydrolyzed in 100 μl of 6*N* HCl at 115°C for 48 hours. The hydrolyzates were dissolved and counted by scintillation spectrometry (*6*). Groups of muscle fibers that were free of end plates were analyzed to determine the correction factor for nonspecific binding (which was about 3 percent of total binding).

From 45 determinations on full-grown male rats (345 to 380 g), the number of ACh receptors ([^{125}I]α-BGT binding sites) per end plate was determined to be (3.98 ± 0.21) × 10^7 (mean ± standard error), and the range was (1.14 to 8.70) × 10^7 sites per end plate. Part of this variability is presumably due to variation in the size of individual end plates. The values determined for rats of various sizes are presented in Table 1. From the binding of [^{131}I]α-BGT to whole hemidiaphragms of rats (weight unspecified), Miledi and Potter (*2*) estimated a population of about 4.7 × 10^7 receptors per end plate.

Table 1. Number of ACh receptors (α-BGT binding sites) per motor end plate in rats (N, number of determinations).

Weight (g) and sex	N	Receptors per end plate	
		Mean ± S.E.	Range
110 ♂*	25	$(1.40 \pm 0.05) \times 10^7$	$(0.91\text{–}1.86) \times 10^7$
180 ♀*	25	$(2.46 \pm 0.08) \times 10^7$	$(1.71\text{–}3.22) \times 10^7$
205–215 ♂♂†	95	$(2.87 \pm 0.10) \times 10^7$	$(1.21\text{–}7.12) \times 10^7$
345–380 ♂♂‡	45	$(3.98 \pm 0.21) \times 10^7$	$(1.14\text{–}8.70) \times 10^7$

* One rat. † Four rats. ‡ Two rats.

The accuracy of these determinations depends on the purity of the [^{125}I]α-BGT and the accurate determination of its specific activity. We isolated α-BGT from lyophilized venom (Miami Serpentarium) by chromatography on carboxymethyl-Sephadex C-50 and rechromatography on Whatman CM-32 carboxymethylcellulose. The purified α-BGT was iodinated to specific activities of (2.34 to 4.86) $\times$ 10^4 curie/mole (average of one iodine atom per molecule of α-BGT) by the chloramine-T method (*7*), and the iodinated product was purified by chromatography on Biogel P-4 and then on carboxymethyl–Sephadex C-50. Protein concentration was determined by the method of Lowry *et al.* (*8*), which was standardized by amino acid analysis. The amino acid composition agrees with that calculated from the known primary sequence of α-BGT (*9*).

We determined the spatial distribution of ACh receptors by examining the distribution of grains in autoradiographs of rat skeletal muscle fibers. The fibers were treated with [^{125}I]α-BGT, washed, and fixed in glutaraldehyde. Lengths of single fibers were dissected out and mounted on slides, which were then dipped in Kodak NTB-2 emulsion. After developing the exposed emulsion, we determined grain density along the fibers and visualized the end plates by staining for AChE. Figure 1 shows typical autoradiographs of single end-plate regions. In A and B, the end plate is located on the upper surface of the fiber in direct contact with the emulsion. In C and D, the end plate is on one side of the fiber.

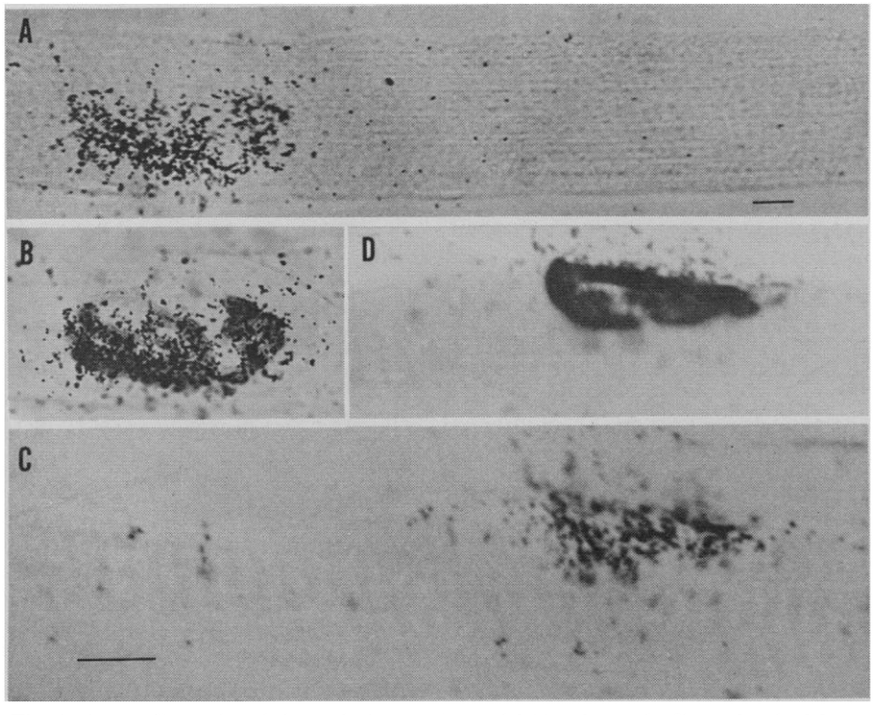

Fig. 1. Autoradiographs of the end plate regions of isolated muscle fibers that had been incubated with [^{125}I]α-BGT. One end plate is shown before (A) and after (B) the preparation was stained for AChE; a second end plate is also seen oriented toward one side of the fiber, before (C) and after (D) staining for AChE. Magnification bars in (A) and (C) represent 10 μm.

The grain distribution (*10*) corresponds quite closely with the synaptic gutters as revealed by AChE staining, and the adjacent muscle fiber surface is comparatively free of grains. From autoradiographs we calculate that in the region outside the end plate but within 130 μm of it, the maximum receptor density is less than 500 sites per square micrometer. Since there may be a small amount of nonspecific binding of α-BGT, we cannot exclude the possibility that there are no ACh receptors outside the end-plate membrane. The estimates of receptor sites per end plate that are based upon grain counts are consistent with the values in Table 1 (although the autoradiographic method is less accurate due to variable sample thickness).

Assuming that the end plate approximates a disk of diameter 25 μm with surface area increased sixfold due to junctional folds (*11*), we estimate the surface area of an average end plate in adult male rats to be about 3000 μm^2. Thus the approximate receptor density in the end plate is 1.3×10^4 receptors per square micrometer. By a comparative autoradiographic method, Barnard *et al.* (*12*) estimated that there are 3.0×10^7 α-BGT sites per end plate and 1.2×10^4 sites per square micrometer for motor end plates in the mouse diaphragm. A similarly dense packing of receptors in postsynaptic membranes in the central nervous system can be inferred from the data of DeRobertis (*13*). In rod outer segments from the retina, where the photoreceptor rhodopsin is most of the membrane protein, receptor density (*14*) is about 2.0×10^4 molecules per square micrometer. By inference motor end plates and other postsynaptic membranes may be constructed largely of receptor molecules. Although accurate data on the surface areas of rat motor end plates are not yet available, it is likely that the receptor density remains fairly constant and that variation in number of receptors per end plate, reported here, reflects heterogeneity in end plate size and enlargement of the end-plate surface area during growth.

We have used [^{125}I]α-BGT in studies correlating receptor density with ACh sensitivity of muscle fibers, measured electrophysiologically. A sensitivity comparable to that at the end-plate (depolarization of several hundred millivolts per nanocoulomb of iontophoretically applied ACh) corresponds

to a receptor density of only about 1.5×10^3 receptors per square micrometer of surface in both the nonendplate regions of adult denervated muscle fibers and in skeletal muscle in tissue culture (*4*). Thus, the receptor density at neuromuscular junctions is much higher than in extrajunctional membranes that are extremely sensitive to ACh. This difference has implications for the appraisal of denervation supersensitivity to ACh and of development and functioning of neuromuscular junctions.

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Morphologic Alterations of Synapses in Electrically Stimulated Superior Cervical Ganglia of the Cat

Abstract. *Prolonged preganglionic stimulation produces marked ultrastructural changes in presynaptic endings, which develop larger zones of contact with postsynaptic dendrites. Profiles of such endings, compared to controls, have fewer synaptic vesicles, similar areas, and greater circumferences. These results are compatible with the hypothesis that synaptic vesicles become incorporated into the plasma membrane during stimulation.*

Biochemical and physiological data suggest that synaptic vesicles may be involved in the mechanism of transmitter release, although direct morphologic evidence is circumstantial (*1*). We investigated the morphology and amount of cholinergic vesicles at neuroneuronal synapses under conditions known to produce marked depletion of neurotransmitter substance. The preparation used was the intensely stimulated superior cervical ganglion of the cat, which had been treated with the drug hemicholinium-3 (HC-3) (*2*). We observed, in addition to changes in numbers of vesicles, unexpected changes in the morphological relation of presynaptic endings to postsynaptic processes in superior cervical ganglia subjected to prolonged electrical stimulation. These data suggest the mechanism of transmitter release that results from stimulation.

Both cervical sympathetic trunks of anesthetized cats were surgically exposed low in the neck, with one side serving as a sham-operated control while the other was placed upon a stimulating electrode. Saline or HC-3 was administered intravenously 5 minutes prior to stimulation. Supramaximal stimulation (as judged by contractions of the nictitating membrane) with monophasic rectangular pulses of 0.3-msec duration and 5- to 10-volt amplitude continued in interrupted trains at 20 to 32 hz for 150 to 190 minutes (*3*). At the end of experiments, stimulation was continued while cats were perfused intraaortically for 10 minutes with an aldehyde fixative (*4*). The ganglia (control and stimulated) were excised, processed simultaneously, and examined by electron microscopy (*5*).

Axodendritic synapses in nonstimulated superior cervical ganglia, in cats treated with saline or HC-3, had a typical ultrastructural appearance. Presynaptic processes were bulbous in shape with a smooth contour, contained spheroidal vesicles, and possessed characteristic membrane specializations (Fig. 1A). Our observations of control ganglia closely agree, qualitatively and quantitatively, with the interrelations reported for axon terminals and dendrites in normal superior cervical ganglia; these interrelations were derived from serial sections examined by electron microscope (*6*).

Visual examination of electron micrographs of synapses in stimulated ganglia, with or without HC-3, disclosed striking alterations in the ultrastructure of a subpopulation of axon terminals (*7*). Profiles of such axon terminals were irregular and crescent-shaped, contained fewer vesicles, had greater zones of apposition with postsynaptic dendrites, and had greater circumferences but similar areas (Fig. 1, B and C). These ultrastructural features apparently resulted from encircling of dendritic profiles by projections of axon endings. In some instances, axon terminals completely encircled dendritic profiles, increasing the zone of apposition to 100 percent. These larger zones of apposition were produced by increases in the nonspecialized portion of the plasma membrane with no obvious increase in the length of synaptic densities. Stimulation did not seem to alter the presynaptic profiles of smallest diameter (0.8 μm).

Measurements were made on electron micrographs in order to specify the extent and frequency of occurrence of the morphological changes. The degree of synaptic apposition for each synapse was expressed as the percentage of the circumference of the dendritic profile in apposition to the axon ending. This measurment was made on random electron micrographs, and frequency distribution histograms were made. The histograms indicated the more frequent occurrence, in stimulated ganglia, of synaptic profiles with a large percentage of the dendritic circumference in apposition to the axon ending. Table 1 shows mean differences between stimulated and control ganglia for this measurement. The effect of stimulation is statistically