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Development of Acetylcholine Receptor Clusters on Cultured Muscle Cells

(α-bungarotoxin/autoradiography/synapses)

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ABSTRACT **Clusters of acetylcholine receptors were formed in the absence of neurons during the development of cultured chick-embryo skeletal muscle cells. The average concentration of receptors in clusters was estimated to be 9000 per μm^2. At other regions of the cell, receptor concentration was 900 per μm^2. The possibility that receptor clusters may participate in synapse formation is suggested.**

[^{125}I]α-Bungarotoxin and related toxins bind to acetylcholine receptors of skeletal muscle grown *in vitro* (1–3). Since the toxins interact with receptors with specificity and relatively high affinity, they have been used to assay both receptor concentration and synapses (1–11). Dividing myoblasts have few, if any, acetylcholine receptors (12). The cells fuse, thereby forming nondividing, multinucleated myotubes with acetylcholine receptors distributed over the entire muscle cell. Upon innervation, active acetylcholine receptors disappear from all parts of the muscle except that portion participating in the synapse (13, 14). Denervation results in the appearance of active acetylcholine receptors over the entire surface of the muscle, starting from the synapse and proceeding towards the cell extremities (15, 16); hence, restriction of receptor location is a reversible process. Similarly, Kuffler, Dennis, and Harris (17) have shown that frog parasympathetic ganglion neurons are highly sensitive to acetylcholine only at the synapse on the cell body, whereas after the incoming axons are cut, the entire surface of the neuron becomes sensitive to acetylcholine.

Acetylcholine receptors may be distributed nonuniformly on cells that have not formed synapses. For example, acetylcholine receptors are restricted to various sites on some mouse neuroblastoma cells (18). Cohen and Fischbach (19) have shown by electrophysiologic methods that muscle cells cultured in the absence of neurons have small areas that are 3- to 6-fold more sensitive to acetylcholine than adjacent areas. Similarly, we have reported that cultured muscle cells have discrete clusters of acetylcholine receptors that bind labeled α-bungarotoxin (3).

In this report, the development and distribution of acetylcholine receptor clusters on muscle cells cultured in the absence of neurons is described, and the formation of receptor clusters is proposed as a normal step in synapse formation.

METHODS AND MATERIALS

Chick-Embryo Muscle Cultures. Cells dissociated from the legs of chick embryos that were 10 days of age were grown *in vitro* by a modification (3) of the method of Konigsberg (20). 50×10^3 Cells were added into each collagen-coated 50-mm petri dish (Falcon Plastics) containing 2.5 ml of growth medium [85% F-14 medium (3); 10% fetal-calf serum (Colorado Serum); 5% chick-embryo extract (Microbiological Ass.)]; 33% of the medium was conditioned, as described by White and Hauschka (21). Cultures were incubated at 37° in a humidified atmosphere of 90% air–10% CO_2; the medium was changed every 3–4 days.

[^{125}I]*α-Bungarotoxin Binding Assay.* [^{125}I]Diiodo-α-bungarotoxin was prepared (2.7×10^5 Ci/mol) by use of a $^{127}I/^{125}I$ ratio of 11.5/1 in the reaction mixture. The diiodotoxin was separated from the monoiodotoxin as described (3). On the assumption that the isotope ratio of the iodinated toxin was the same as that of the reaction mixture, about 2% of the diiodo-α-bungarotoxin molecules were labeled with two ^{125}I atoms; 12% with one ^{125}I and one ^{127}I; and 86% with two ^{127}I atoms.

Before incubation of cells with labeled α-bungarotoxin, the medium was removed from each petri dish and replaced with 1.5 ml of solution A [the Dulbecco-Vogt modification of Eagle's medium (22) (GIBCO) containing 20 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid), pH 7.4, in place of $NaHCO_3$, and 2 mg of crystalline bovine-serum albumin (Armour Pharm. Co.) per ml of medium]. Cultures were incubated at 37° for 20 min in air, then [^{125}I]diiodo-α-bungarotoxin was added (3.3 mM final concentration) and cultures were incubated for an additional 60 min. The medium was removed and each culture was washed three times with 3-ml portions of solution A and three times with 3-ml portions of solution A without albumin. Cells with bound toxin were removed and radioactivity was determined (3).

Autoradiography. Cells incubated and washed as described above were fixed for 1 hr in a solution containing 2.5% glutaraldehyde and 0.1 M sodium phosphate buffer, pH 7.4 (2 ml per culture) at 24°; then each culture was washed six times with 2 ml of 0.1 M sodium phosphate buffer, pH 7.4 (10 min per wash) and dehydrated by adding in succession 3 ml of 35, 50, 75, 95, and 100% ethanol (5 min per step). Dishes were dried in air, coated with Kodak NTB-2 emulsion diluted 1:1 with H_2O, and incubated at 4° for 4 days in a light-proof box containing a desiccant. Autoradiographs were developed for 4 min with Kodak D-19 developer at 26° and fixed with Kodak Fixer.

RESULTS

The rate of appearance of acetylcholine receptors on cultured chick-embryo muscle cells is shown in Fig. 1. Cells divided rapidly during the early stages of culture. Myoblast fusion was first observed on the third day of incubation, resulting

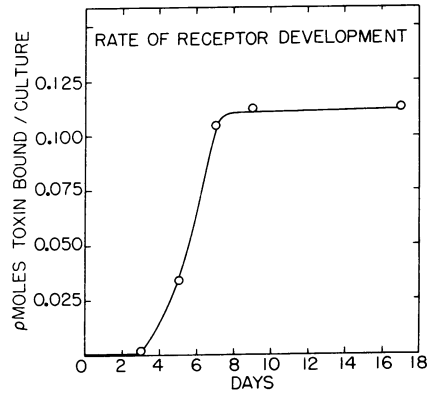


FIG. 1. Binding of [^{125}I]diiodo-α-bungarotoxin to chick-embryo skeletal muscle grown *in vitro* for different periods. Cells were incubated with 3.3 nM [^{125}I]diiodo-α-bungarotoxin, as described in *Methods*. Each point represents the average value found with duplicate 50-mm petri dishes (each dish, 20-cm^2 surface area). Sister cultures were subjected to autoradiography (shown in the following figures).

in the formation of nondividing, multinucleated myotubes and, concomitantly, a marked increase in acetylcholine receptors was observed. The maximum number of receptors per culture was achieved by the eighth day of incubation, and the number of receptors per culture remained constant thereafter.

During the course of this experiment, replicate cultures were subjected to autoradiography. A phase-contrast view of cells cultured for 3 days is shown in Fig. 2, panel *A*, and the corresponding autoradiograph is shown in panel *B*. Multinucleated myotubes formed by cell fusion were labeled with α-bungarotoxin, whereas mononucleated cells, such as myoblasts and fibroblasts, were not labeled (panels *C* and *D*). An occasional mononucleated cell bound α-bungarotoxin, but such cells usually were not heavily labeled (panels *E* and *F*). In Fig. 3, panels *A* and *B*, are shown phase-contrast and bright-field views of muscle cells cultured for 5 days and subjected to autoradiography. Many myotubes were present, and all were labeled with α-bungarotoxin. Acetylcholine receptors were distributed rather uniformly over the entire surface of each myotube; however, some myotubes contained more receptors than others. No unlabeled myotubes were found in this or other experiments.

After 7 days of incubation, discrete clusters of acetylcholine receptors were present on about 1% of the myotubes (Fig. 4, panels *A*, *B*, and *C*). In Fig. 5 autoradiographs are shown of cells cultured for 9 days (panel *A*) and 11 days (panels *B* and *C*). Cells cultured for 9 days had more receptor clusters than younger cultures, and by the 11th day in culture, more than 80% of the myotubes had receptor clusters. Some myotubes had as many as 20 clusters. The size of receptor clusters varied considerably; the average cluster was 125 μm^2. They were found predominantly along cell margins and were frequently ovoid in shape, the long axis of the cluster being parallel to the long axis of the cell, and boundaries were defined rather sharply. Regions of cells that were richest in receptors had larger and more frequent clusters; however, they were also found on myotubes that were labeled relatively lightly with α-bungarotoxin (see bottom of panel *B*). Occasionally, receptor clusters were found on structures that resembled partially retracted processes. The clusters

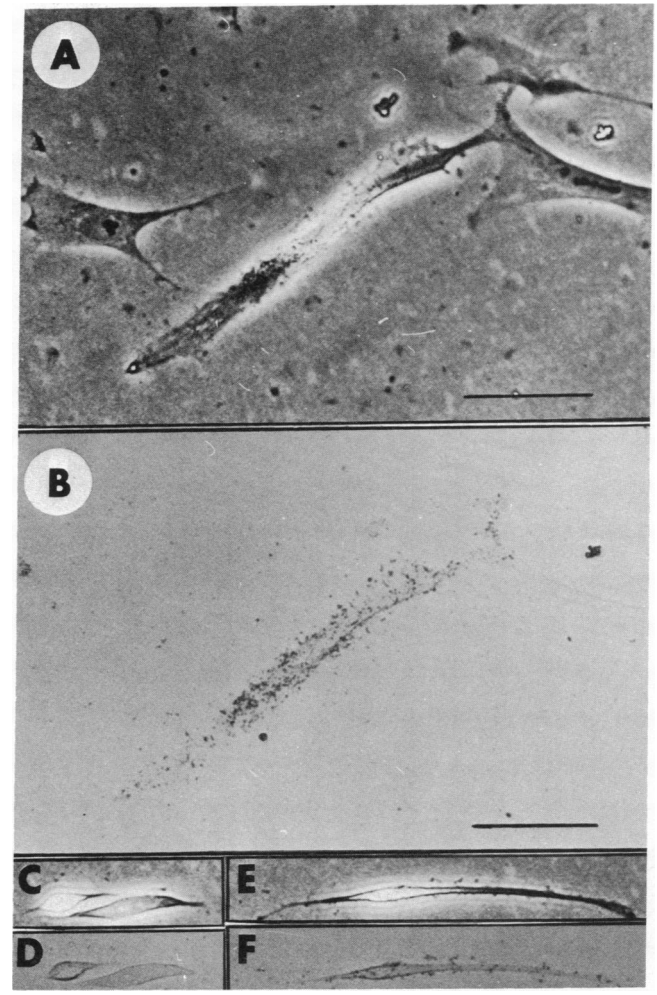


FIG. 2. Autoradiographs of chick-embryo muscle cells cultured for 3 days and then labeled with [^{125}I]diiodo-α-bungarotoxin (see Fig. 1). Panels *A*, *C*, and *E* are phase-contrast views to show cell detail; panels *B*, *D*, and *F* are the corresponding autoradiographs viewed with bright-field optics to show silver grains. The *bar* represents 50 μm and the scale applies to all panels.

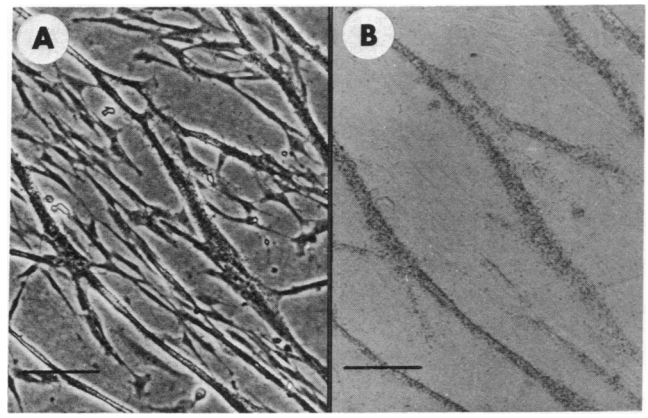
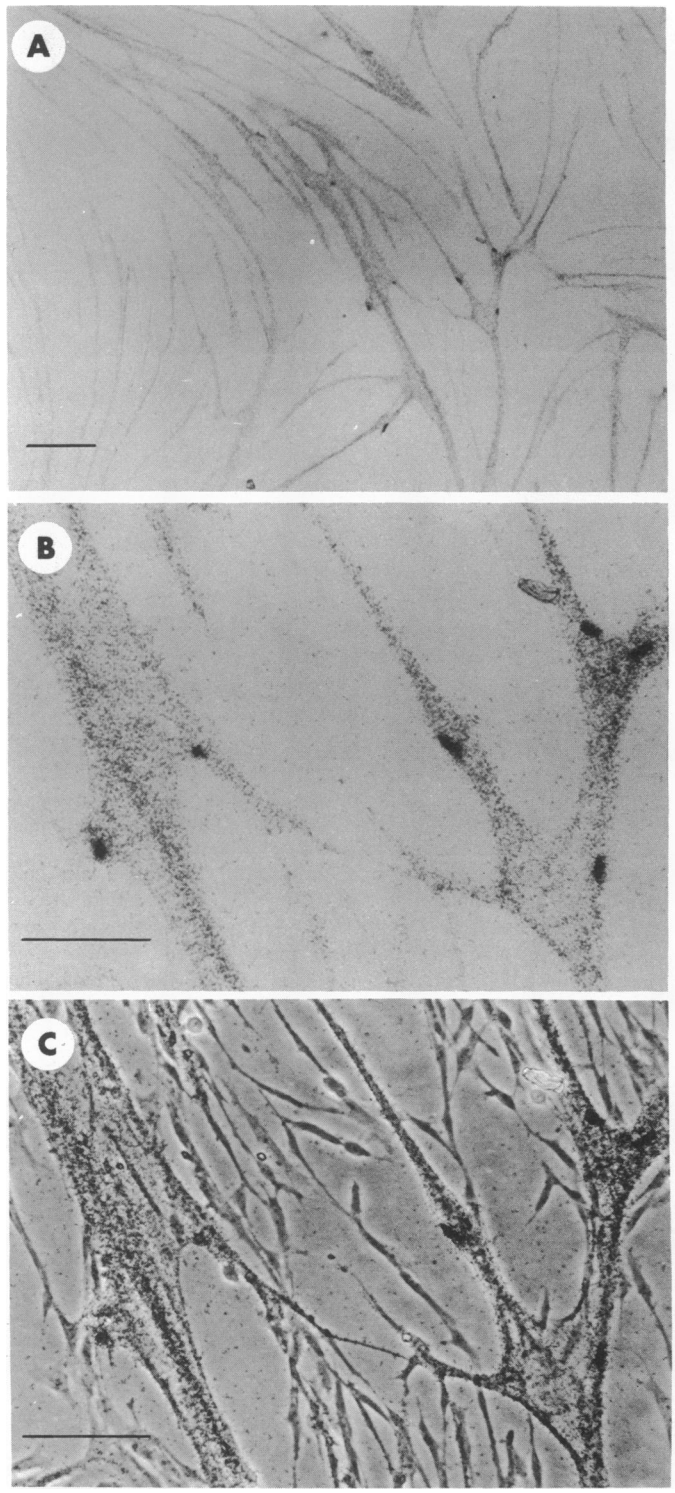


FIG. 3. Autoradiographs of muscle cells cultured for 5 days and then labeled with [^{125}I]diiodo-α-bungarotoxin (see Fig. 1). Panel *A* is a phase-contrast view and panel *B* the corresponding autoradiograph. The *bar* in each panel represents 100 μm.

were not consistently associated with a cell organelle when viewed with phase-contrast optics. Similar results were obtained in preliminary electron microscopic studies.

The clusters, as well as diffuse labeling of myotubes, were also found when cells were incubated in F-14 growth medium or with [^{125}I]monoiodo- instead of [^{125}I]diiodo-α-bungarotoxin (3). Similar results were obtained when other methods of fixation were used, such as (*a*) 100% methanol; (*b*) 2.5%



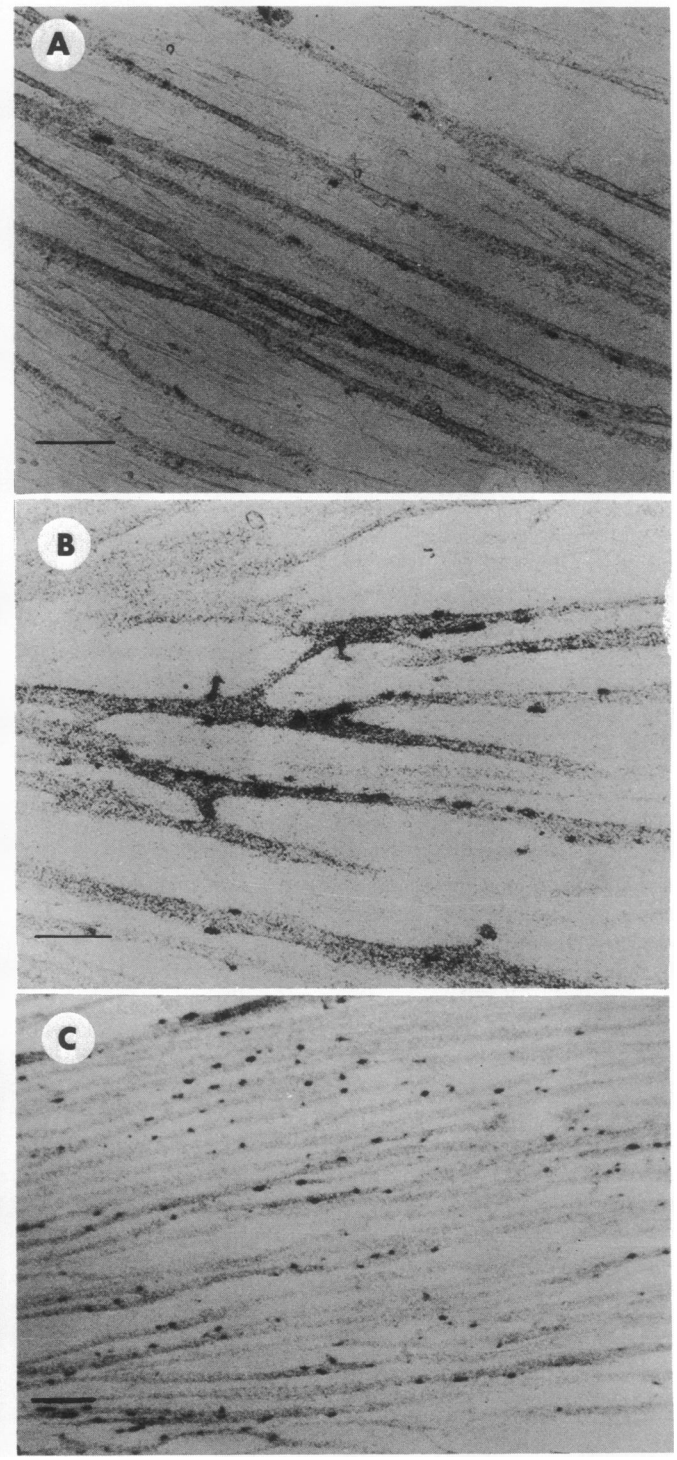


FIG. 4 (*left*). Autoradiographs of muscle cells cultured for 7 days and labeled with [^{125}I]diiodo-α-bungarotoxin (see Fig. 1). In panels *A* and *B*, photomicrographs obtained with bright-field optics are shown at low and high magnification, respectively. The *bars* in panels *A* and *B* represent 200 and 100 μm, respectively. In panel *C*, a phase-contrast view of the field corresponding to panel *B* is shown; the *bar* represents 100 μm.

FIG. 5 (*right*). Autoradiographs of muscle cells labeled with [^{125}I]diiodo-α-bungarotoxin (see Fig. 1). Panel *A* is a bright-field view of cells cultured for 9 days. Panels *B* and *C* are bright-field views of cells cultured for 11 days. The *bar* in each panel represents 100 μm.

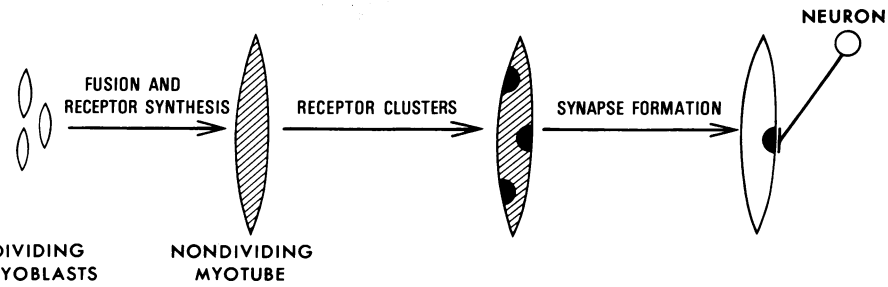


FIG. 6. Formation of acetylcholine receptor clusters, a possible step in synapse formation. Dividing myoblasts fuse and form nondividing multinucleated myotubes with acetylcholine receptors uniformly distributed over the surface of the cell (step 1). Synapse-like clusters of acetylcholine receptors then form in the absence of neurons (step 2). Acetylcholine receptors or molecules associated with them may interact with appropriate molecules on the innervating neuron, and thus may specify synapse formation. Synapse formation is accompanied by the disappearance of active acetylcholine receptors from the noninnervated portion of the muscle cell surface, thereby converting the muscle cell from a permissive to a nonpermissive state with respect to establishing functional synapses with other neurons.

glutaraldehyde–0.1 M sodium cacodylate (pH 7.4)–2 mM $CaCl_2$; or (*c*) 10% formaldehyde in Dulbecco's phosphate-buffered saline (pH 7.4) (23). Cells fixed for 45 min in a solution containing 10% formaldehyde in Dulbecco's phosphate-buffered saline before incubation with labeled α-bungarotoxin bound 80% of the amount of toxin bound to unfixed cells, and no change was observed in diffuse or clustered receptor distribution.

Cells were subjected to autoradiography, embedded in Epon 812, sectioned, and examined with an electron microscope. The results showed that the NTB-2 emulsion was distributed in a uniform thickness over the myotubes and remained in contact with the cell membrane. The distribution of acetylcholine receptors on the cell surface in contact with the petri dish was examined as follows: after incubation cells were fixed with glutaraldehyde and embedded in Epon 812 while still attached to the petri dish. Each dish was removed, and NTB-2 emulsion was applied to the surface of the Epon formerly attached to the dish. Autoradiographs revealed that uniformly distributed and clustered receptors were present on the bottom surface of the cells.

In other experiments fewer cells were added to each petri dish to obtain well-isolated muscle colonies. All myotubes in the 101 colonies examined bound α-bungarotoxin, and every colony cultured 11 days or longer had receptor clusters.

In other experiments autoradiographs were exposed for only 20 hr in order to determine the number of silver grains per cluster. From the specific activity of the labeled α-bungarotoxin, and on the assumation that one molecule of toxin binds to one acetylcholine receptor, the average receptor concentrations of the diffusely labeled and cluster regions of the cell were estimated to be 900 and 9000 receptors per μm^2, respectively.

DISCUSSION

The results show that clusters of acetylcholine receptors form during the development of chick-embryo skeletal muscle cells cultured in the absence of neurons. On the assumption that one molecule of α-bungarotoxin binds to one acetylcholine receptor, the concentration of receptors in the clusters was about 9000 per μm^2, similar to that found at the neuromuscular and the electroplax synapse (7, 8, 10, 24, 25). The average receptor concentration in other regions of the cell was 900 μm^2.

It is unlikely that the receptor clusters are artifacts of fixation, since no apparent relation was found between the abundance of clusters and the method of fixation, and such clusters were not observed on chick-embryo sympathetic ganglion neurons grown *in vitro* that were assayed in the same way (L. Greene *et al.*, manuscript in preparation). In addition, Cohen and Fischbach examined living muscle cells grown *in vitro* by electrophysiological techniques and found small areas of cells 3- to 6-fold more sensitive to acetylcholine than adjacent areas (19).

The data suggest that some receptor clusters result from retraction of cell processes. It should be noted that because of the close apposition of the lower and upper cell membranes, the grain concentration at the cell margin may be twice that found towards the cell center. However, the concentration of silver grains in receptor clusters was considerably more than twice that of neighboring regions. No ultrastructural specialization of surface membranes or cell organelles were associated with these regions.

Formation of receptor clusters may be due to interactions between acetylcholine receptors and other molecules. Acetylcholine receptors of electroplax apparently interact either with each other or with other molecules to form more complex structures (6, 26–28).

A possible relation between receptor clusters and synapse formation is shown schematically in Fig. 6. Dividing myoblasts have few, if any, active acetylcholine receptors, whereas nondividing, multinucleated myotubes have many receptors distributed over the entire cell surface. Regions of relatively high acetylcholine receptor concentration appear on muscle grown *in vitro*. Synapse formation *in vivo* is accompanied by a loss of active acetylcholine receptors from the noninnervated portion of a muscle cell, thereby converting the muscle cell from a permissive to a nonpermissive state with respect to establishing functional synapses with the neurons (16, 29, 30).

It seems likely that molecules attached to the tip of an exploring axon interact with complementary molecules attached to the surface of another cell and thereby select sites for synapse formation. Obviously a cell receiving information across a chemical synapse must have an appropriate species of receptor and other components of the neurotransmitter translation apparatus. Such molecules or molecules that interact with them may be part of a synaptic recognition

code. Stable interaction between two cells almost surely depends upon many molecular interactions. It is possible that acetylcholine receptors or molecules associated with them function as determinants of synapse recognition, and that receptor cluster formation may be a normal step in synapse formation. Further work is needed to determine whether such clusters are required for synapse formation.

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