# Brain Extract Causes Acetylcholine Receptor Redistribution Which Mimics Some Early Events at Developing Neuromuscular Junctions

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ABSTRACT We studied the effect of rat brain extract on rat muscle cells in vitro by light and electron microscope (EM) autoradiography after labeling acetylcholine receptors (AChR's) with $^{125}$I-$\alpha$-bungarotoxin. We found that: (*a*) In the absence of brain extract, peak site densities within AChR clusters usually do not exceed 4,000 sites/$\mu m^2$. (*b*) Within hours after exposure to brain extract, AChR's redistribute to form clusters in which the peak site densities are >10,000 sites/$\mu m^2$. Receptor concentration within extract-induced clusters is thus within a factor of 2 of that at the neuromuscular junction (nmj). (*c*) In the absence of extract, the AChR's and AChR clusters are predominantly on the bottom surface of the myotubes (facing the tissue culture dish). After extract treatment, they are predominantly at the top surface. (*d*) Plasma membrane in regions of high-density AChR clusters is enriched in membrane with enhanced electron density and surface basal lamina whether or not cells are treated with extract. Extract causes an increase in both these specializations on the top surface of the myotubes. (*e*) Brain extract does not produce an overall increase in AChR site density or a marked change in degradation rate of receptors in either clustered or nonclustered regions. By producing AChR clusters with junctional site densities and enhanced surface specialization, and by causing an overall shift in AChR's distribution, brain extract mimics early events reported at developing neuromuscular junctions.

When muscle cells in culture are innervated, there is a major change in the distribution of surface acetylcholine receptors (AChR's), resulting in a high-density accumulation at the postsynaptic membrane (1, 18, 24). This accumulation of AChR does not require neuromuscular transmission (6, 43, 45) and involves, in part, a redistribution of existing receptors (1). It is reasonable to suggest that factors released from the nerve may be responsible for the initial clustering of AChR's at developing neuromuscular junctions (nmj's). Studies have shown that soluble extracts of nervous tissue or conditioned medium from nerve cultures can in fact alter the concentration and distribution of AChR's on muscle cells in vitro (4, 15, 25, 38, 47). The changes demonstrated in these studies varied somewhat for different muscle systems but involved an increase in the number of high-density AChR clusters (4, 15, 25, 38, 47) and in some systems an increase in overall AChR number (25, 38). It is not yet obvious, however, how these changes are related to the events occurring during synaptogenesis. We were interested in determining whether, and in which way, the response to neural extract resembles any of the events which occur during the formation of the nmj.

By using quantitative light and electron microscope (EM) autoradiography after labeling AChR's with $^{125}$I-$\alpha$-bungarotoxin ($^{125}$I-BTX), we confirmed an earlier observation that, in the absence of neuronal influence, receptor clusters on rat primary muscle cells in vitro usually do not exceed ~3,000–5,000 sites/$\mu m^2$ (28). We found, however, that after these cells are treated with rat brain extract, the peak receptor concentration within individual AChR clusters can reach site densities approaching those at the nmj (>10,000 sites/$\mu m^2$). We also found that the extract-induced receptor clustering does not require a decrease in turnover rate and can be produced by a redistribution of existing receptors. In these respects it resembles events reported at developing nmj's (1, 10, 24, 43, 44, 45). Our data thus suggest that a soluble factor(s) released from the ingrowing nerve could cause the accumulation of AChR seen at the nmj.

## MATERIALS AND METHODS

The number and distribution of AChR was monitored by the binding of $^{125}$I-

BTX. The validity of this approach for labeling the nicotinic AChR on vertebrate skeletal muscle has been established in numerous studies (e.g., reference 18).

## Preparation of Cells and Extract

Primary skeletal muscle cultures were prepared from thigh muscle of fetal rat (20 ± 1 d of gestation). Muscle was dissected free of skin and bone, minced, and then dissociated in 0.2% trypsin (Sigma Chemical Co., St. Louis, MO) in $Mg^{2+}$ and $Ca^{2+}$-free Earle's balanced salt solution. After the cells were washed free of trypsin by centrifugation, they were plated into 100-mm tissue culture dishes (Falcon Labware, Oxnard, CA) in Dulbecco's modified Eagle's solution (DME) + % fetal calf serum (FCS) for 30 to 45 min at 37°C. The cells that remained unattached during this time were transferred to collagen-coated 35-mm tissue culture dishes (Corning Glass Co., Corning, NY) containing 2 ml of DME + 10% FCS. Cytosine-1-$\beta$-D-arabinofuranoside (Ara C) (final concentration $10^{-5}$ M) was added to the cells 4 d after they were plated to kill rapidly dividing cells (21). In the absence of Ara C, the cultures tended to be overgrown by fibroblasts which made grain counting over whole myotubes very difficult. In addition, preliminary results suggest that the response of individual myotubes to extract was somewhat decreased in the absence of Ara C. The reason for this and the possible role of fibroblast over-growth in this observation remain to be established.

Brain extract from fetal rats, of 19 ± 2 d gestation, were prepared in Puck's saline G (PSG) exactly as described previously (38). Cells received one or two doses of 100 $\mu$l of extract containing ~9.0 mg/ml protein in PSG between days 4 and 6 postplating, as indicated.

After extracts were centrifuged at 100,000 *g*, they were sterilized by filtration through 0.45-$\mu$m Millipore filter, and stored frozen at −20°C. Extracts were used within 7 d of their preparation and were thawed no more than twice. Extracts lost activity after two freeze-thaw cycles. We observed no particulate material larger than the detection limit of 5 $\mu$m before or after storage, nor did any seem to form after extract was added to the cells. Cells were labeled with $^{125}$I-BTX ($2 \times {}^{-8}$ M; 40–1,000 Ci/mmol) for 40 min at room temperature in Tyrode's solution pH 7.4 + bovine serum albumin (BSA), (2 mg/ml) followed by a series of rinses in Tyrode-BSA for a total of 1 h. In those experiments where the cells were not fixed until 1 to 3 d after labeling, the original growth medium was placed back onto the cells after the last rinse period.

In rat myotubes the binding of $^{125}$I-$\alpha$-BTX is correlated with ACh sensitivity (28) and is blocked by $10^{-4}$ M d-tubocurarine or $10^{-7}$ M $\alpha$-BTX. Background binding either over mononucleated cells or bare dish was <10% of that on myotubes (see also references 28, 38). Saturation of AChR was shown by varying the concentration of $^{125}$I-$\alpha$-BTX and the duration of incubation followed by gamma counting and autoradiography (28).

## Light Autoradiography

Quantitative light autoradiography was performed using a monolayer of Ilford L4 emulsion calibrated for thickness and sensitivity to iodine-125 as previously described (28). Care was taken to avoid saturating the emulsion over clusters by using different exposure times for different dishes from the same labeling condition. AChR site densities for clusters were calculated only from dishes with grain densities within the linear range for the emulsion (<0.6 grains/$\mu m^2$).

The developed autoradiograms were examined with a Reichert Diapan microscope using anoptral phase-contrast objectives. Myotubes were examined under low magnification (without seeing the distribution of developed grains in the emulsion layer) and were chosen for analysis only if they could be seen from end to end, unobstructed by other myotubes and cells. A minimum of four random myotubes were counted per 35-mm dish, and care was taken to pick these from different areas of the dish. Grains were counted under oil immersion × 100 objective. A grid in the ocular (1 square = 38 $\mu m^2$) was aligned along the length of the myotube, and grains were counted along the full length of the myotube in a strip which was one square wide and chosen so as to include the receptor clusters whenever possible. All the grains were summed and an average value per square was obtained. The grain clusters were then identified by an arbitrary definition as any square which had a grain density three times above the average value. Grain densities were separately recorded in each of four categories: average

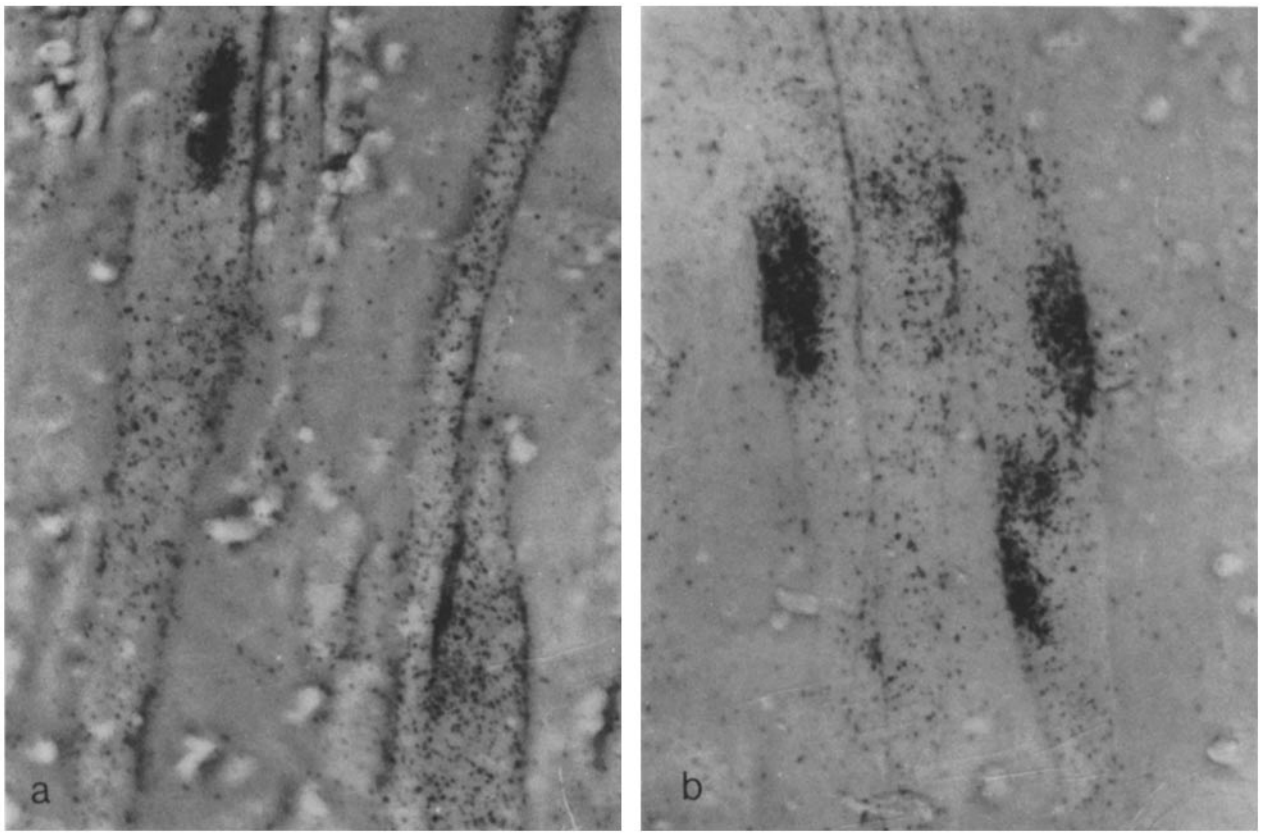



FIGURE 1 Extract effect on overall distribution of $^{125}$I-BTX binding. Light microscope autoradiograms show nonextract-treated cells (*a*) and cells after 1 d in extract (*b*). Note increased receptor clustering after extract treatment. Autoradiograms are overexposed, for illustration, and thus cannot demonstrate the increased receptor site density seen in clusters after extract treatment. × 800.

TABLE I

*Effect of Extract on AChR Site Density on Primary Rat Muscle Cells by Light Autoradiography*

| Time after labeling | Treatment | AChR site density (sites/$\mu m^2$)* | | | Ratio CI/IC1 | % Area occupied by cluster |
|---|---|---|---|---|---|---|
| | | Average | Cluster‡ | Intercluster | | |
| *d* | | | | | | |
| (*a*) 0§ | No extract | 895 ± 105 | 3,519 ± 360 | 724 ± 128 | 5.7 ± 1.3 | 5 ± .9 |
| | Extract | 1,534 ± 149 | 8,860 ± 797 | 726 ± 95 | 16.2 ± 2.9 | 10.1 ± .9 |
| | Ratio X/NX | 1.7 | 2.5 | 1.0 | 2.8 | 2.0 |
| (*b*) 1‖ | No extract | 238 ± 56 | 1,033 ± 191 | 204 ± 51 | 6.9 ± 1.6 | 4.5 ± 1.4 |
| | Extract | 304 ± 48 | 1,631 ± 238 | 186 ± 37 | 11.4 ± 1.7 | 8.6 ± .6 |
| | Ratio X/NX | 1.3 | 1.6 | .9 | 1.7 | 1.9 |
| (*c*) 3¶ | No extract | 16 ± 6 | 82 ± 6 | 13 ± 5 | 6.7 ± 1.6 | 4.2 ± 1.1 |
| | Extract | 45 ± 4 | 360 ± 27 | 18 ± 6 | 19.0 ± 9 | 7.7 ± .9 |
| | Ratio X/NX | 2.8 | 4.4 | 1.4 | 3.4 | 1.7 |

AChR site densities decrease with time after labeling with a half-life <1 d; calculated using the equation $t_{1/2} = -\ln 2\ [t/\ln (R/I_o)]$ where R = residual label after time $t$ and $I_o$ is initial label.

* Values are mean ± SE for about five myotubes from each of three to five dishes per condition. *X*, extract treated; *NX*, nonextract treated; *CI*, cluster; *ICI*, intercluster region.

‡ A cluster is defined as any tabulation grid square (38 $\mu m^2$) in which the site density is more than three times the average value. This definition was chosen since it correlated well with visually identifiable "hot spots," which ranged in size from one to three contiguous grid squares. Since the grain density was often higher within a fraction of such a grid square, all the "cluster" values are slight underestimates. Occasionally nonextract treated myotubes had no clusters by our definition. Such myotubes were included in the average value but not in the cluster of intercluster values.

§ Extract treatment involved adding extract on days 4 and 5; cells were labeled and fixed on day 7 (data as in Fig. 2).

‖ Cells were labeled on days 4 or 6, with or without a simultaneous single addition of extract, and fixed one day later.

¶ Cells were labeled on day 4 (with or without addition of extract on days 4 and 5) and fixed on day 7.

over the whole tube, average per grain cluster, average for intercluster regions, and the highest square per myotube (called peak value). A background value, obtained in an area next to each myotube, was subtracted. The site density of AChR per $\mu m^2$ was then calculated from this grain density by the equation given in Land et al. (28).

## *EM Autoradiography*

Cells were prepared for fine structure and EM autoradiography as previously described (28). We used pale gold (1,000 Å) tissue sections, deep purple (monolayer) films of Ilford L4 emulsion, and D19 development (2 min, 20°C). Four randomly selected myotubes were sampled from each of four dishes. Developed autoradiograms were scanned and overlapping photographs taken so that a montage of an extended region of the myotube could be analyzed.

Absolute quantitation of BTX binding sites (AChR sites) per surface area of membrane was calculated from the grain density and specific activity of the BTX as previously described (28), but, unlike the previous study, where only intercluster site densitites were tabulated, we also analyzed receptor site densities within clusters. Because of the difficulties in sampling the full length of a myotube by EM autoradiography, our criterion for what constitutes a cluster was somewhat different than that used to analyze our light autoradiograms. An AChR cluster in EM autoradiograms was any length of membrane, containing more than five grains, whose average site density was at least five times higher than that on an equal length of membrane on either side of that cluster. (In this definition of a cluster, the grain density is compared only to neighboring membrane and not to the whole myotube. Since the grain density within intercluster regions is not uniform, the cluster/intercluster ratio from our EM autoradiograms can be <5.) Because of the sharp gradient in receptor concentration at the interface between cluster and intercluster regions, our criteria for what constitutes a cluster by light and EM atuoradiography did identify clusters with similar site densities.

## RESULTS

The first experiment by light autoradiography involved labeling and fixing the cells after 1 or 3 d in the presence of extract (between days 4 and 7 in vitro). As previously reported, AChR clusters were seen in nonextract-treated myotubes (22, 28, 46), and extract increased the average number of clusters (~1.8-fold) (4, 15). This, plus an ~1.5-fold increase in the average area per cluster, resulted in a two- to threefold increase in surface area of myotube occupied by clusters (Fig. 1, Table I *a*). A new finding was the dramatic effect of extract treatment on increasing the AChR site density within clusters (Fig. 2).

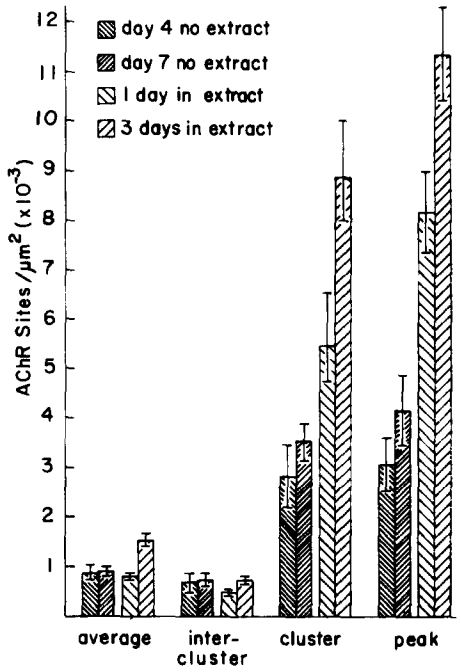



FIGURE 2 Histogram of AChR site densities by light microscope autoradiography, representing the top surface of myotubes tabulated in the four categories as described in Materials and Methods. Based on seven experiments, 47 myotubes, and 10,600 developed grains. For cells 1 d in extract, the extract was added on either days 4 or 6, and the cells were labeled and fixed 1 d later. For cells 3 d in extract, extract was added on day 4 and again on day 5. The cells were labeled and fixed on day 7. The percent area occupied by clusters was ~3-6% in the absence of extract and ~7-10 d after extract.

Extract also caused the "peak" density (i.e., the tabulation grid square with the highest site density in each myotube) to reach values that are within a factor of two of that seen at postnatal nmj's (11, 19, 32, 34, 40). After 3 d in extract, the average peak

site density from all the myotubes was ~11,000 ± 1,000. Peak values of >20,000 were encountered in individual myotubes, and 55% of myotubes had at least one tabulation square with a site density >10,000 sites/$\mu m^2$. In the absence of extract, the peak values were 3,000–5,000 sites/$\mu m^2$ and no myotubes had any tabulation square with a site density over 10,000 sites/$\mu m^2$.

Data in Fig. 2 also show that after 3 d in extract there is an increase of about twofold in the average receptor site density over the entire myotube surface. Thus we found that extract treatment caused a formation of high-density clusters and an increase in overall receptor site density. In the experiments to follow, we asked whether the formation of clusters was due to a redistribution of pre-existing receptors, and what might cause the increase in overall site density. Three nonmutually exclusive possibilities exist for explaining the increased site density: (*a*) extract causes an increase in AChR synthesis; (*b*) extract decreases the metabolic degradation rate of AChR; or (*c*) extract causes labeled receptors to be introduced from a source not detected by the emulsion in the light autoradiograms. Such a source could be the bottom of the cells since Ilford L4 emulsion which was used in our light autoradiographic studies has been shown to be exposed by $^{125}I$ only within a range of ~2,000 Å (20). Our light microscope autoradiograms thus provide information only for the top surface of the myotubes.

To test whether clusters can form by a redistribution of existing receptors and whether the increased site density reflected an increase in newly synthesized receptors, we labeled AChR's with $^{125}I$-$\alpha$-BTX before exposure to extract and then kept the cells, with or without extract, for 1 d or 3 d before fixing for light autoradiography. We found that within 1 d in extract (Table I*b*) there was a redistribution of prelabeled receptors into clusters, giving a ratio of cluster-to-intercluster site density similar to that seen when receptors were labeled after exposure to extract (Table I*a* and Fig. 2). In one experiment the redistribution of receptors was seen as early as 3 h in extract but more often it occurred by 8 h.

We also found that even with the prelabeled receptors there is a more than twofold increase in overall receptor site density after 3 d in extract (Table I*c*). Therefore, an increase in receptor synthesis could not explain the higher site density seen on the top surface of the cells by light autoradiography. EM autoradiographic studies allowed us to examine the distribution of AChR at the top and bottom of the myotubes (Fig. 3). We found that 3 d in extract caused a shift in AChR concentration

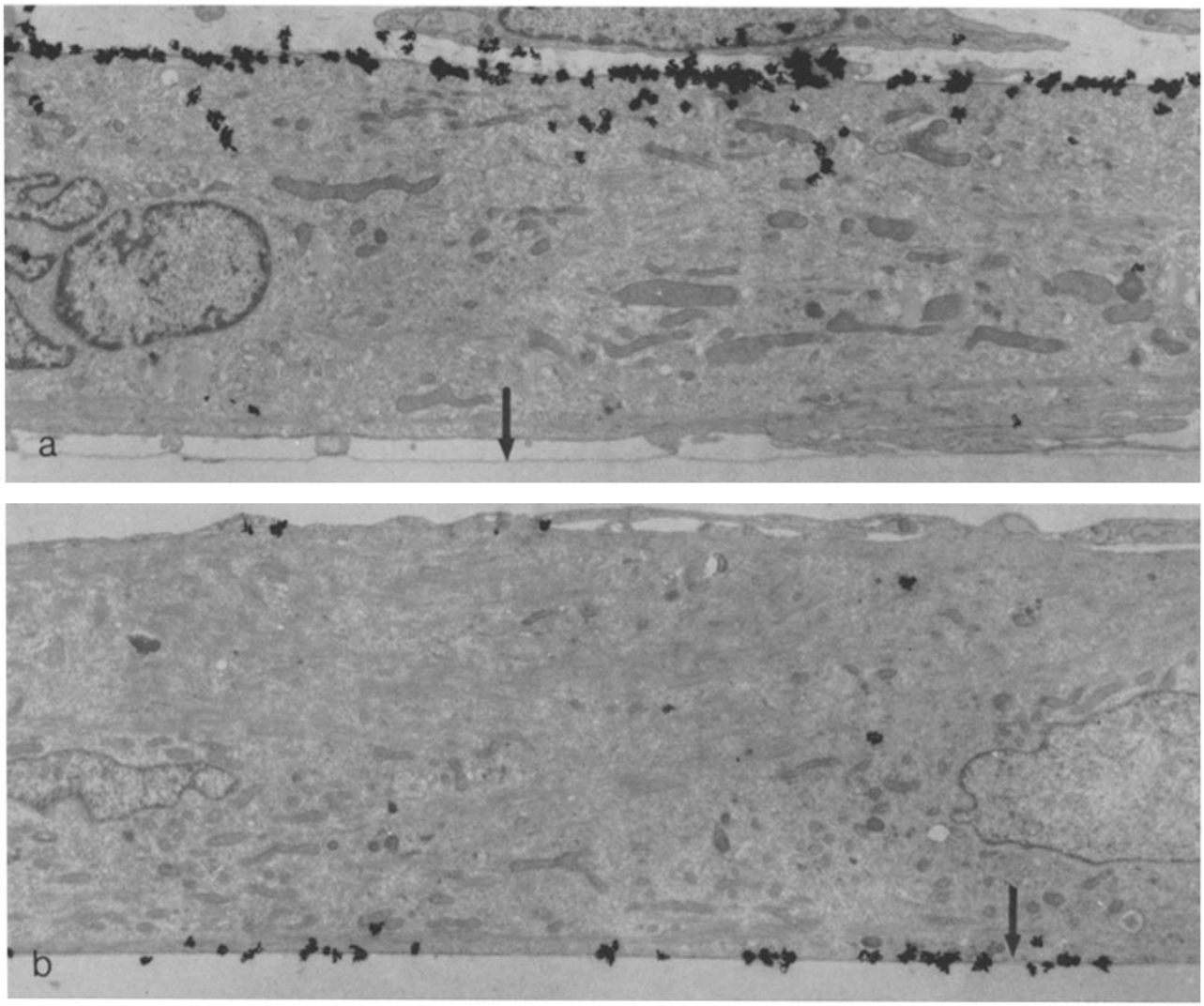



FIGURE 3 Overexposed EM autoradiograms show typical localization of receptor cluster on top surface after extract treatment (*a*) and at the bottom surface in nonextract-treated cells (*b*). Bottom of cell can be identified by characteristic line (arrow) derived from the tissue culture dish. × 5,000.

from the bottom to the top of the cell. Earlier studies have reported that receptors and receptor clusters tend to favor the bottom of the myotubes (3, 7, 28), and that extract causes clusters to appear at the top surface of myotubes (2, 38). In this study we analyzed such observations quantitatively and found that, in the absence of extract, there is a two- to threefold higher average receptor concentration (Fig. 4) and five- to sixfold larger area occupied by clusters at the bottom of the myotube than at the top. The percent area of membrane which contained clusters in the absence of extract was ~2% at the top and ~11% at the bottom of the cells. After 3 d in extract, the top surface has a three- to fourfold higher average receptor site density (Fig. 4) and a threefold larger area occupied by clusters. (Approximately 8% of the area had clusters at the top vs. 3% at the bottom.) It is of interest that even though there were fewer clusters at the bottom of the myotube after extract, the site density within clusters was elevated to the same extent at the bottom as at the top. The overall AChR site density for the whole myotube (top plus bottom) did not increase in response to extract (Fig. 4). The overall number of receptor clusters decreased by ~40%. We suggest that a redistribution of receptors from the bottom of the cells to the top in response to extract could quantitatively account for the increase in average receptor density observed with light autoradiography. This conclusion is supported by our determination of the amount of BTX bound specifically to myotube cultures (using scintillation or gamma counting) and the surface area of myotubes in these cultures (using the grid method of Chalkley [13]). We found that, although extract sometimes causes a 30–40% higher BTX binding per dish, it was accompanied by a similar increase in the myotube surface area.

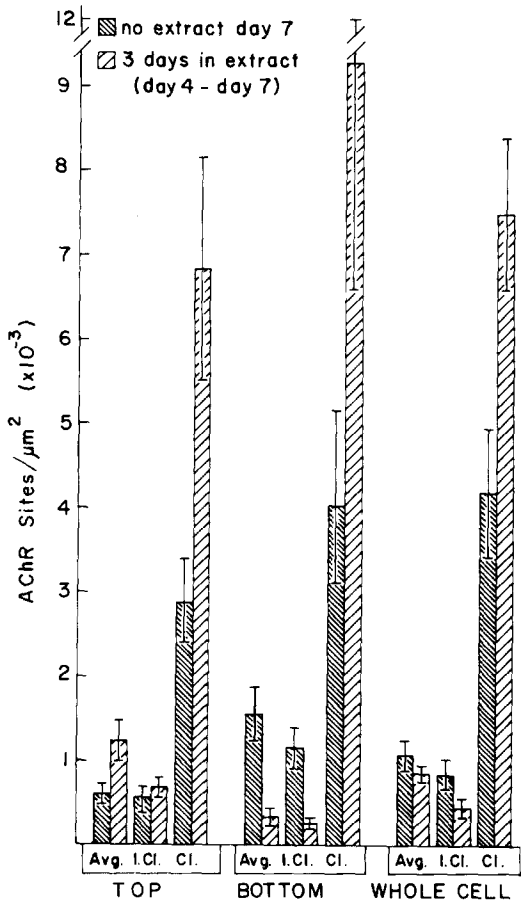
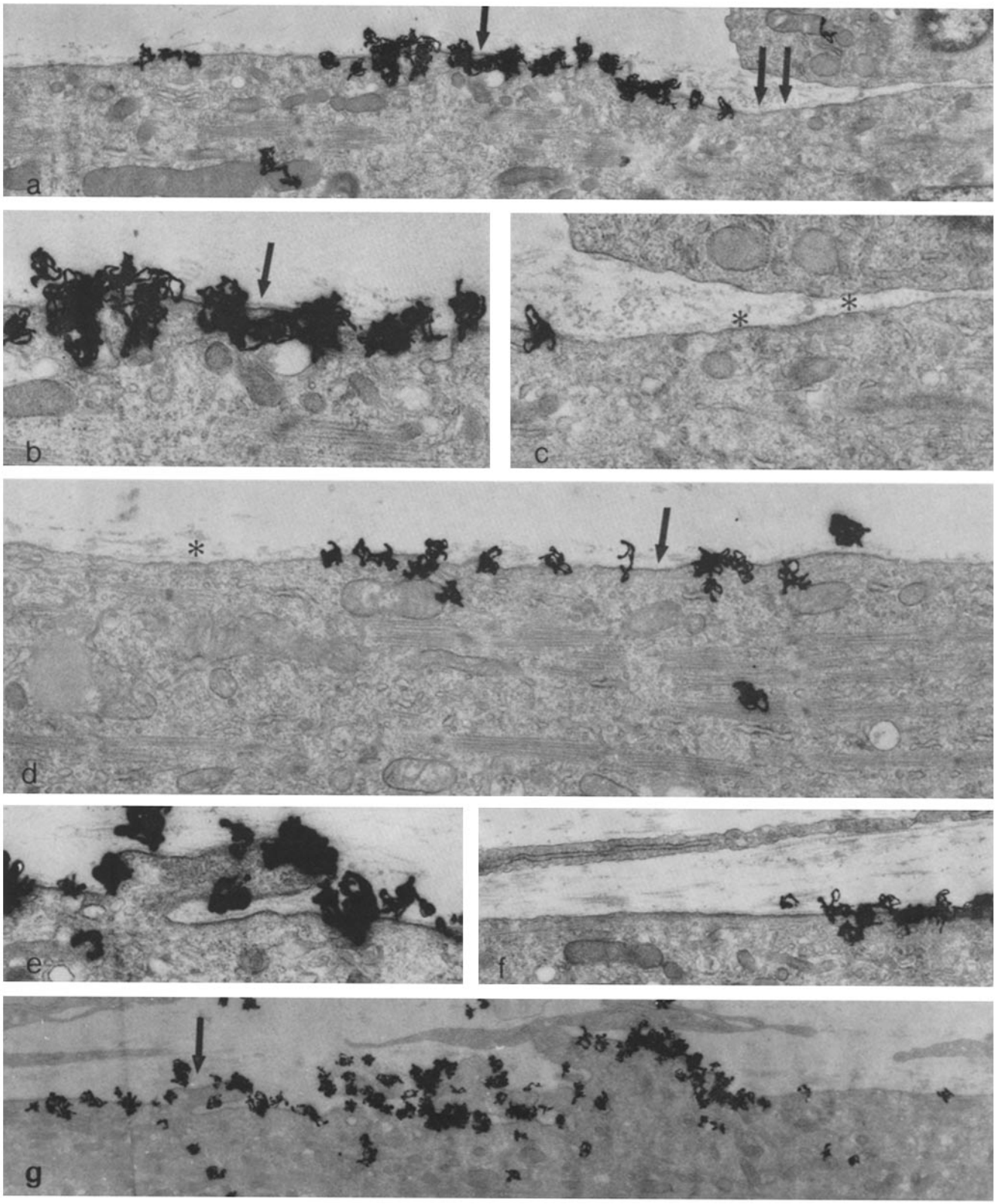



FIGURE 4 Histogram of AChR site densities by EM autoradiography. Based on eight myotubes, 1,800 developed grains, and >7,000-μm length of myotubes examined. Extract was added on days 4 and 5, and the cells were labeled and fixed on day 7. For each surface (top, bottom, or whole cell), AChR site densities were either averaged (*Avg*) or tabulated separately for cluster (*Cl*) or intercluster (*ICl*) regions. The site densities of AChR at the top surface seen by EM autoradiography are similar to that seen by light autoradiography (Fig. 2), indicating that the smaller sample used for the EM autoradiography was statistically valid. Note that, although the average and intercluster AChR site densities at the bottom of the tube decreased in response to extract, the density within clusters increased as it did at the top. We found, however, that the number of clusters at the top increased while those at the bottom decreased (see text).

We estimated the degradation of AChR by the label remaining on the myotube with time after labeling with $^{125}$I-BTX (17, 18) using light autoradiography. If we assume an exponential degradation rate of AChR, we can calculate an approximate half-life ($t_{1/2}$) by comparing the label remaining on day 7 (3 d after labeling) with that seen immediately after labeling on day 7 (Table I). We used day 7 for our zero time so that both sets of cells would have had a 3-d exposure to extract and thus have the same extent of receptor redistribution from the bottom of the cell. The validity of using day-7 cells for our zero label requires, as shown by our data (Fig. 2), that the overall receptor number in the absence of extract on days 4 and 7 is similar, and that redistribution into clusters is the same for prelabeled and newly synthesized receptors (Table I). We found that the calculated $t_{1/2}$ for both extract- and nonextract-treated cells is ~13 h for both clustered and intercluster receptors. Thus, extract does not cause a marked change in degradation rate of AChR's at the top surface of the myotubes, at least within the 3 d used in our studies.

Our examination of the EM autoradiograms was extended to include finestructural specializations which are characteristic of the nmj: electron density of plasma membrane and the presence of basal lamina. By using the EM autoradiograms, we were able to examine separately regions of membrane with and without receptor clusters, at both the top and bottom surfaces of myotubes. The judgment regarding the morphological specialization is subjective, but images such as are illustrated in Figs. 5 and 6 were considered as representing increased electron density and fragments of basal lamina. "Dense membrane" was plasma membrane which appeared thicker, more rigid, and often contained a cytoplasmic fuzzy layer. "Basal lamina" was a continuous surface layer separated from the plasma membrane by a less electron-dense zone. Since we did not use fixation and staining procedures designed to preserve and enhance the basal lamina, and since both dense membrane and basal lamina would be missed in tangential sections and could be obstructed by the developed grains in our autoradiograms, no exact values can be given. Furthermore, on the underside of the cells, dense membrane is also characteristic of contact zone between cells and the dish, (e.g., Fig. 6) and basal lamina could not be visualized in regions where the cells are in close contact with the dish. However, on the top surface of the cells, the relative values, comparing cluster and intercluster regions with and without extract treatment, indicate a valid trend that requires consideration. We found (Figs. 5 and 6) that membrane regions containing clustered receptors were enriched (more than fourfold) with both electron-dense membrane and basal lamina. However, neither dense membrane nor basal lamina fully coexisted with clustered regions. This was true whether or not cells were treated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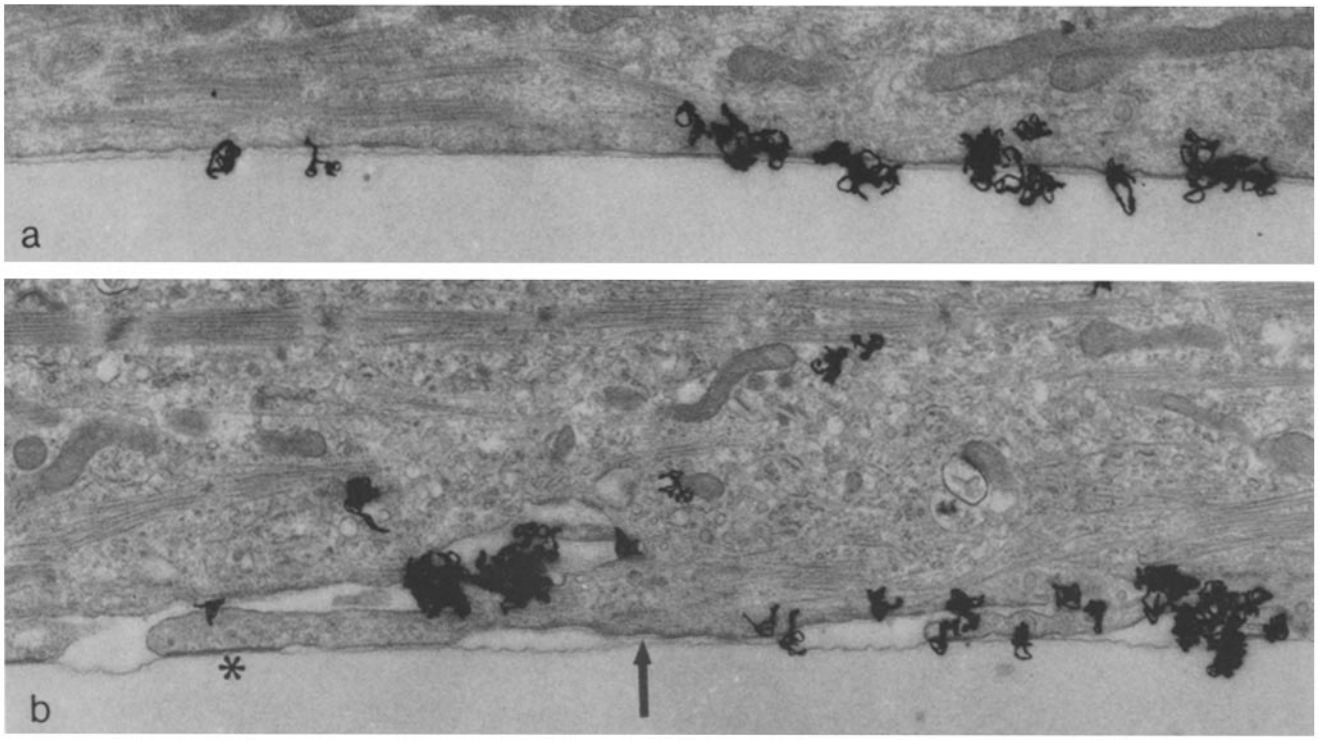


FIGURE 6 AChR cluster regions at bottom of cell in nonextract-treated cell (*a*) and extract-treated cell (*b*). Note that dense membrane is prominent at contact zones between cell and dish (asterisk) which is not necessarily associated with receptor clusters. Dense membrane and basal lamina (arrow) are prominent but not present throughout the cluster region. × 15,000.

with extract. Furthermore, the dense membrane in clusters tended to be continuous, whereas in nonclustered regions the dense membrane was more often in the form of small discontinuous bits (Fig. 5). The dense membrane specialization and the basal lamina in myotubes are similar to that seen in cells co-cultured with nerve (16, 36) and in neonatal endplates (J. Matthews-Bellinger and M. M. Salpeter, manuscript in preparation). The cytopalsmic "fuzz" is somewhat less intense than that seen at the postsynaptic membrane of adult nmj's (19).

In extract-treated cells, clusters were often associated with membrane bulges (Figs. 5 and 6). Most interesting, extract caused an overall increase in both dense membrane and basal lamina at the top surface of the cells and within clusters (about two- to threefold) paralleling the increase in AChR's and AChR clusters. Extract did not cause any notable change in cellular differentiation as judged by myofibrillar organization (see Figs. 5 and 6), nor did it affect the cytoplasmic label which has been reported when intact embryonic or denervated muscle cells are labeled with α-BTX (28, 32, 39, and Matthews-Bellinger and Salpeter, manuscript in preparation).

## DISCUSSION

The major results of this study are: (*a*) Brain extract can induce the formation of high-density AChR clusters with peak site densities approaching junctional values. (*b*) The high-density clusters can form by a redistribution of pre-existing receptors within 1 day. (*c*) There is a shift of receptors from the bottom to the top of the cell within 3 d in extract. And (*d*) there is also an increase in the relative amount of dense membrane and basal lamina on the top of the cell. In one respect, primary rat myotubes are different from those of the cloned line $L_6$ and from the chick, both of which show an overall increase in receptor site density as well as an increase in clustering (25, 38). The reason for this difference is not clear. In our earlier study (38), we suggested that the increase in receptor sites density on $L_6$ cells may be due to the initially low site density

FIGURE 5 Overexposed EM autoradiograms of extract-treated cells showing top surface of different cells: (*a*) characteristic AChR cluster on slight membrane bulge. Area within cluster (arrow) enlarged in *b* and from adjacent intercluster region (double arrow) enlarged in *c*. Note: basal lamina overlying electron-dense membrane in cluster region (arrow in *b*) compared with intercluster region without basal lamina and with dense membrane in small bits (asterisks in *c*). "Basal lamina" by definition was a continuous line separated from the plasma membrane by a less dense zone. "Dense membrane" was characterized by being thickened, more electron dense, and more "rigid" in appearance than the rest of the membrane, and was often associated with a cytoplasmic "fuzz." (*a*) × 12,400. (*b* and *c*) × 24,800. (*d*) Top surface containing continuous dense membrane and basal lamina (arrows) in clusters, small bits of dense membrane (asterisk) and essentially no basal lamina, in intercluster region. Note also characteristic alignment of myofilaments with only rudimentary or no Z band. The sequence of differentiation in primary rat myofibers is thus similar to that reported previously for $L_2$ cells (27, 39). We found no accelerated or altered sequence in myofilament differentiation in response to extract. × 16,300. (*e, f,* and *g*) Overexposed autoradiogram of clustered regions (*e* is enlargement of region marked by arrow in *g*). Note: membrane bulges (*e* and *g*), dense membrane, and basal lamina in the cluster region (*e* and *f*). The dense membrane and basal lamina extend a short distance beyond edge of cluster (*f*). Note grains in cytoplasm in *a, d,* and *g*, representing a diffuse internal label as previously reported (28). (*e*) × 22,400. (*f*) × 13,900. (*g*) × 7,400.

in these cells. It would be of interest to establish whether different neural factors are involved in controlling site density and clustering. Because the rat myotubes respond only with clustering, this aspect has become the focus of our study.

The fact that receptor clusters form on muscle cells both in vivo and in vitro in the absence of nerve (8, 22, 46) and may form in response to several nonphysiological conditions (26, 37) has suggested that the initial formation of clusters may be independent of neural control. By means of autoradiography, we have demonstrated what was undetected by the fluorescence technique (2), that brain extract causes the receptor concentration within clusters to approach that seen in the presence of nerve. In contrast, at the only nonphysiological site at which an AChR site density has been determined (i.e., the myotube/dish interface, examined in this study), clusters have a density no higher than that seen generally in the absence of nerve. Thus, there may be a general tendency for AChR's to cluster spontaneously or in response to a wide variety of stimuli, but neuronal factors appear well suited to mobilize and enhance this tendency.

One possible mechanism which could produce a shift in receptor concentration from the bottom to the top of the cell could be a change in the relative number of mobile versus immobile AChR's. For example, if in the absence of extract there were a higher fraction of immobile receptors at the bottom of the cell and after extract treatment there were a higher fraction at the top, then, once the mobile receptors have equilibrated, one would see a shift in receptor site density from the bottom to the top, as we have reported. With a diffusion constant of $10^{-10}$ $cm^2/s$ (3) or faster, and a myotube of 30 $\mu m$ in diameter, mobile receptors would equilibrate well within the 3-d exposure to extract used in our study. An increase in the fraction of immobile receptors at the top of myotubes has been reported in rat muscle cells exposed to conditioned medium from neuroblastoma/glioma hybrids (2). It remains to be established whether there is a larger number of immobile receptors at the bottom of the myotube in the absence of neuronal factors. The mechanisms which could account for the redistribution of AChR's to form clusters may also involve local regions within which receptors are immobilized. In addition, since the shift of receptors from bottom to top is accompanied by a net loss of clusters, but not of AChR's, there may be a translocation and coalescence of whole clusters. Alternatively, there may be a dispersal of AChR's from clusters into the mobile pool followed by a redistribution.

Immobilization of receptors or receptor clusters may involve specializations such as are reflected in membrane electron density with its associated cytoplasmic "fuzz" and/or basal lamina (12), both of which are affected by extract and are normally enriched in clustered regions. Dense membrane may merely reflect a high protein content in the membrane or anchoring filaments. The increase (about threefold) in basal lamina in response to extract and the reported role of basal lamina in localizing AChR clusters in denervated regenerating muscle (12) raise the question of whether basal lamina may not serve as an "intermediate step" in the extract-induced clustering phenomenon, possibly by immobilizing receptors. Rubin et al. (42) have reported that preparation of particulate material of basal lamina can cause AChR clusters to form on chick myotubes in vitro. It remains to be determined whether such clusters have the elevated site densities which are seen after treatment with brain extract.

Any final interpretation of the role of dense membrane and basal lamina in the extract-induced cluster phenomenon must consider that, although receptor clusters are enriched in dense membrane and basal lamina, we found that clusters need not have either dense membrane or basal lamina, and that both dense membrane and basal lamina exist in intercluster regions. Such a nonmandatory coexistence between dense membrane and AChR was also described in the fetal mouse at nerve-muscle contacts during a period when AChR site densities have not yet reached adult values (Matthews-Bellinger and Salpeter, manuscript in preparation; see also reference 36). At present, we must emphasize that we have no evidence which would relate any morphological specialization to the extract-induced effect on AChR. Our results on basal lamina and dense membrane are intriguing, however, and encourage further investigation.

Several characteristics of AChR's have been linked to various stages of development of the nmj's. The first is the clustering of AChR at the nerve-muscle contact regions which involves, at least in part, a migration of preexisting receptors (1). Subsequently, the receptors become stabilized anatomically, retaining their localization on the postsynaptic membrane even after denervation (31, 41). Other maturation changes occur with innervation such as metabolic stability (5, 11, 14, 29–31, 44) and a decrease in gating time of AChR channels (23, 35), and changes in isoelectric point (9) and immunological properties (48). It is now clear that these different steps in the development and maintenance of the nmj are independently controlled, and the challenge is to unravel the diverse manner in which a nerve can participate in these events.

In this study, we were concerned with the first step, that of receptor clustering and migration. It is often assumed that clustering does not require the nerve. However, since receptor clusters seen in the absence of nerve or nerve factors do not reach junctional concentrations, it now appears that one more step in synaptogenesis, the acquisition of high AChR density within clusters at the developing nmj, is possibly under neural control. We show that a soluble neural factor could be sufficient to accomplish the migration of AChR and its clustering to junctional values, if such a factor is indeed released at the developing nmj in vivo.

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