Proc. Natl. Acad. Sci. USA
Vol. 75, No. 4, pp. 2035—2039, April 1978
Neurobiology

Nerve extract induces increase and redistribution of acetylcholine receptors on cloned muscle cells

(neurotrophic phenomena/α-bungarotoxin binding/L_6 cells)

T. R. PODLESKI*, D. AXELROD†‡, P. RAVDIN*§, I. GREENBERG*, M. M. JOHNSON*†, AND M. M. SALPETER*†

* Section of Neurobiology and Behavior and † School of Applied and Engineering Physics, Cornell University, Ithaca, New York 14853

Communicated by Leon A. Heppel, January 12, 1978

ABSTRACT **The effect of rat spinal cord explants and cell-free nerve extract on acetylcholine receptor site density and distribution was studied using ^{125}I- and rhodamine-labeled α-bungarotoxin on L_6, a cloned rat muscle cell line. Control L_6 myotubes have a low and uniform distribution of acetylcholine receptors (20 ± 3 sites per μm² in the present study). The addition of spinal cord explants caused an increase in average receptor site density of about 6 times on myotubes within 2 mm of the explant, while a smaller increase of 3 times was observed at distances greater than 5 mm. The formation of high-density patches of receptors was also stimulated. These observations suggested that a diffusible substance originating from the explant was responsible for these changes. Cell-free homogenates of the central nervous system were prepared and found to produce the same effects. The effect of the homogenate was not strongly dependent on the age of the fetus from which the tissue was isolated, and fetal liver had little or no effect. The active component(s) appears to be a protein(s) with a molecular weight of about 100,000. Because the nerve homogenates make the L_6 cells resemble primary muscle cultures, we suggest that a common factor is responsible for regulating the acetylcholine receptor in the two types of muscle culture. The normally acetylcholine receptor-poor L_6 cells may provide a more sensitive assay for these factors than do primary muscle cultures.**

Many studies on neurotrophic phenomena have dealt with the control of synthesis and distribution of the acetylcholine receptors (AcChR) in embryonic and adult innervated and denervated muscle. One favored system for these studies has been muscle cells grown in culture [see recent reviews by Nelson (1) and Gutman (2)].

In primary muscle cultures the AcChR tends to form in high density patches on myotubes. Several authors have suggested that these patches play a role in the subsequent development of the cells (3, 4).

The L_6 line of cloned muscle cells (5) resembles primary muscle cultures in its developmental sequence and ability to form synaptic connections (6). Furthermore, the conductance change per AcChR in response to AcCh is the same in L_6 as in primary rat myotubes (7). However, these cells have a uniform distribution and considerably lower site density of AcChR than the myotubes in primary cultures (7–9). These characteristics make L_6 a desirable system for studying the possible control of AcChR distribution in skeletal muscle prior to innervation.

The present study deals with the effects of isolated explants of nervous system tissue and of cell-free nerve extracts on AcChR in L_6 cells. We have found that both are capable of inducing an increase in average AcChR site density and the formation of high-density patches.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

METHODS

The acetylcholine receptors defined as α-bungarotoxin (BTX) binding sites were visualized either by fluorescence after labeling with rhodamine-α-bungarotoxin (R-BTX) (10, 11) or by quantitative autoradiography after labeling with [^{125}I]iodinated α-bungarotoxin (^{125}I-BTX) (9). For both probes the specificity of toxin binding was tested by competition with unlabeled toxin or 0.1 mM *d*-tubocurarine. Incubation with ^{125}I-BTX under the conditions of these experiments has been shown to inhibit the response to iontophoretically applied acetylcholine (7). For the quantitative autoradiography, cells were fixed in 2% (vol/vol) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4), washed in distilled water, and air dried before being coated with emulsion.

In our earlier studies (ref. 7 and unpublished observations) we had found that primary fixation with glutaraldehyde caused considerable membrane blebbing and poor preservation of fine structure, especially for mononucleated cells. These effects were eliminated if 0.1% OsO_4 was added to the glutaraldehyde. However, the AcChR site density was not significantly different with or without the OsO_4 addition to the glutaraldehyde.

In order to avoid problems due to the uneven drying of liquid emulsion over the nonuniform surface of the cells in culture, we used a monolayer stripping film of Ilford L_4 emulsion. Sensitivity was assessed using known sources of ^{125}I; chemography was controlled with an evaporated carbon layer (7). Developed grains were counted under high-dry optics (Reichert Diapan, "anoptral" phase contrast) at ×640. Regions of myotubes to be counted were chosen randomly by focusing on three to four arbitrarily chosen widely dispersed regions of each dish, then moving the stage left until the nearest myotube was encountered. Grains were then counted in 8–24 adjacent unit areas, each consisting of 100 μm² over this randomly encountered region of the myotube. (Myotubes were omitted only if dirt obstructed the counting view.) We estimate that depending on the size of the myotube, we counted grains over 10 to >50% of the entire myotube surface area. AcChR sites per μm² were then calculated from the counted grain density, using the formula given previously (7, 12) for which an accuracy of better than ±30% is estimated. In addition to the randomly selected myotubes each dish was systematically scanned to locate one myotube with a very high, and one with a very low, AcChR density so as to assess the range of site density in one dish.

Abbreviations: AcChR, acetylcholine receptor(s); BTX, α-bungarotoxin; R-BTX, rhodamine-conjugated BTX.

‡ Present address: Biophysics Research Division, Institute of Science and Technology, University of Michigan, Ann Arbor, MI 48105.
§ Present address: Department of Biology, University of California, San Diego, B-022, La Jolla, CA 92032.

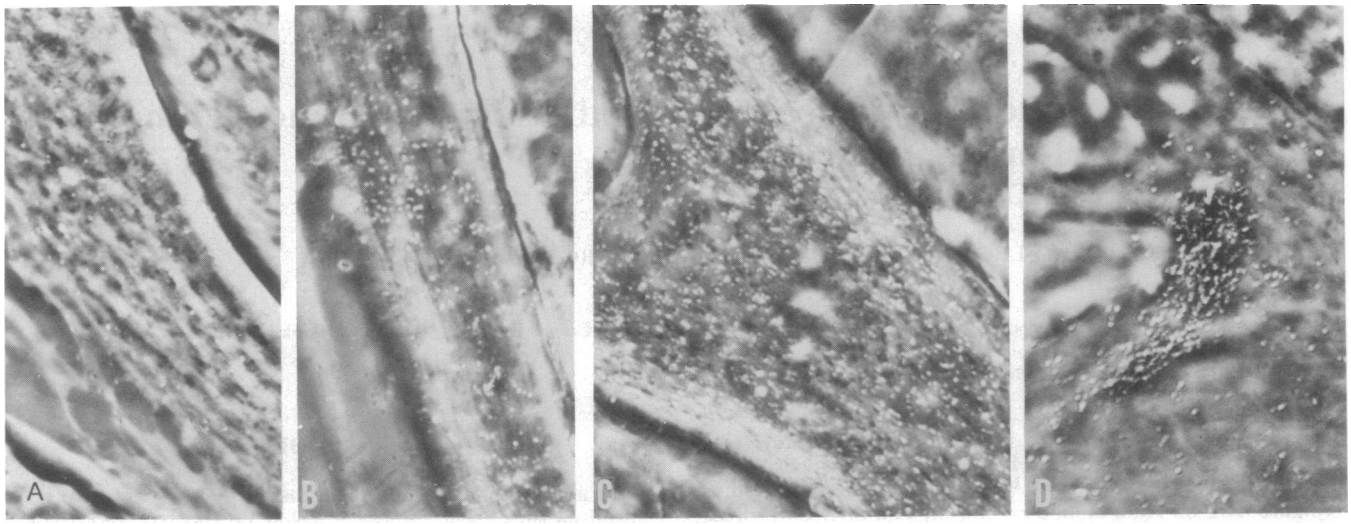

FIG. 1. Autoradiograms of L_6 myotubes treated with ^{125}I-BTX and photographed by using "anoptral" phase contrast (Reichert Diapan). Under these conditions developed grains look phase bright. (×960.) (*A*) Untreated control. (*B*) Treated with brain extract. (*C* and *D*) Near a spinal cord explant. Note high density patch in the fork of a large myotube (*D*).

Nerve extracts and explants were made from 18-day-old rat fetuses, unless otherwise indicated. To make nerve extract, tissues were homogenized in a glass hand homogenizer in Puck's saline G (pH 7.2) and centrifuged at 500 × *g*; the supernatant fraction was removed, and the pellet was rehomogenized in saline G and recentrifuged, all at 4°. The two supernatant fractions were pooled and sonicated for ≃20 sec., frozen overnight, and then centrifuged at ≃100,000 × *g* for 60 min. The supernatant fraction of the 100,000 × *g* centrifugation was assayed for protein by the method of Lowry, using bovine serum albumin as a standard (13). The total volume of material applied daily was kept constant (a maximum of 140 μl/day) by supplementing volumes with saline G.

Spinal cord explants were cut into approximately 1-mm cross sections and placed on muscle cultures. The muscle cells had been plated into the culture dish 4 to 5 days before explants or extracts were added. L_6 cells were plated at a density of 5×10^4 cells per ml into 35-mm Corning tissue culture dishes. Conditions for growing these cells have been published elsewhere (7). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum. The volume of Eagle's medium used was 2 ml, and this medium was not changed during the course of the experiment. The cells grow exponentially for about 4 days; they begin to fuse on day 4 or 5 and continue to fuse during the next 2 or 3 days.

We tested several regimens for adding extracts to the muscle cultures. The procedure that produced the optimum formation of high-density patches of AcChR was the application of extract beginning on the day fusion started, i.e., on day 4 or 5, and continuing at daily intervals for the next 2 days. In all cases the growth medium with extracts remained on the cells until the time the cells were incubated in BTX. Normally this occurred within 2 days after the last application of extract. Procedures for treating cells with R-BTX or ^{125}I-BTX have been published previously (7, 10) and involved incubation in 0.1 μM BTX for 1 hr at room temperature, followed by a 1-hr wash in Tyrode's solution plus bovine serum albumin at 2 mg/ml prior to preparation for either viewing with fluorescent microscope or fixing and preparing for autoradiography.

RESULTS

Nerve explants and cell-free nerve extracts caused both an increase in AcChR (BTX) site density and the formation of high-density patches of AcChR. Figs. 1 and 2 show photographic examples of treated and untreated L_6 cells when labeled with either R-BTX or ^{125}I-BTX, and Tables 1 and 2 summarize the results obtained with different extracts and explants. The fluorescent technique provided a rapid qualitative measure of the presence of high-density AcChR patches, but intrinsic background fluorescence made detection of AcChR outside patches difficult. The fluorescent technique was thus not very sensitive in detecting increases in average AcChR site density. R-BTX-labeled myotubes showed high-density receptor regions ≃12–20 μm long, whereas cells treated with control buffer showed few if any such patches. In general, nerve extract- or explant-treated myotubes also showed a very dim diffuse labeling between patches, which was most easily seen by a tangential edge brightening. Most patches were near the edges of the myotubes, some were on the top, very few were on the bottom. This is in contrast to observations made on primary rat muscle cultures, where most of the patches were found on the bottom of myotubes (7, 10), and predominantly where myotubes were in contact with the tissue culture dish (7). The patches on both primary and L_6 cells have a granular internal structure with a final structural unit of less than 1 μm^2 (see Fig. 2). The persistence of this patch substructure over periods of time of at least 10 min indicates that the AcChR within these regions is relatively immobile (10).

Using the R-BTX, we found no difference between the extracts prepared from brain or spinal cords and could not distinguish between 14- and 20-day-old fetuses when equal amounts of protein were used. As Table 1 shows, extracts prepared from adult central nervous systems were also capable of causing patches of receptors to form. Amniotic fluid and liver extracts prepared from the same fetuses as the central nervous system extracts and matched for protein content resulted in erratic responses: occasionally patches were formed but generallly these materials were significantly lower in activity than extracts prepared from the central nervous system.

By light microscope autoradiography (Table 2) we were able to both determine the AcChR average site density of untreated L_6 cells and assess the extent to which the nerve extract affected the distribution of bound toxin. We found that nerve extract and explants caused the formation of high-density patches, but the predominant appearance, when scanning dishes prepared for autoradiography, was the increase in the overall receptor

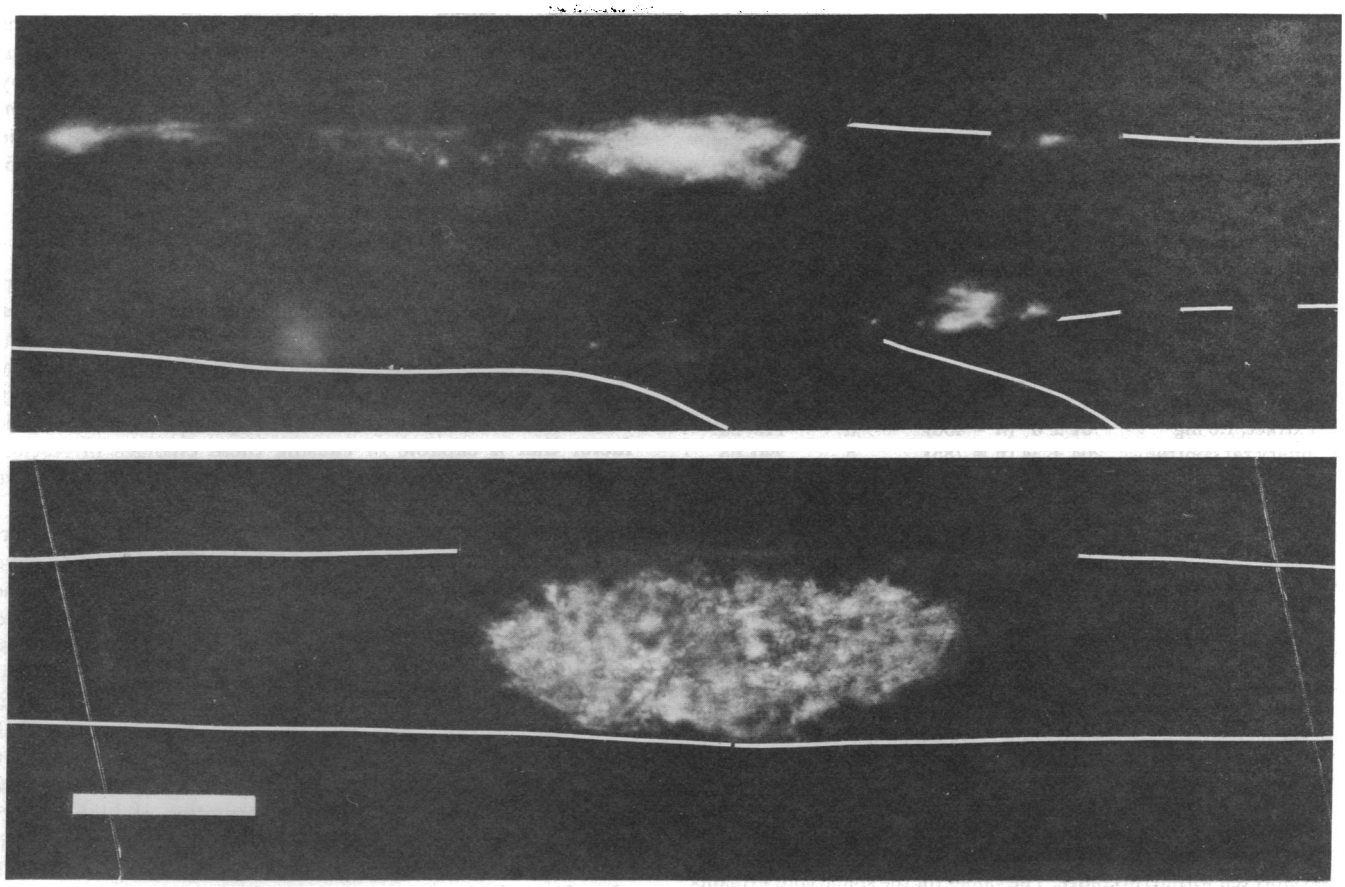

FIG. 2. Acetylcholine receptor patches on myotubes visualized by fluorescence of R-BTX. (*Upper*) L_6 myotube treated with spinal cord extract. (*Lower*) Rat primary myotube with no extract added. The outlines of the myotubes are depicted by drawn white lines. Calibration 20 μm.

site density. The control level of AcChR site density depends on the clone of L_6 cells used. In the present study the average control site density on randomly chosen myotubes from four experiments ranged from 5 to 33 sites per μm^2 and was thus at the lower end of the site density range previously reported using other L_6 clones (7). However, with both the fluorescent probe and autoradiography, we saw basically a similar response to various treatments in several different L_6 clones. After treatment of the L_6 cells with either cell-free nerve extract or spinal cord explant, there was a 3 to 6-fold increase in average AcChR site density, depending on the particular treatment. In addition, the site density of occasional high-density patch regions (averaged over 100 μm^2) was as high as 600 AcChR sites μm^2. Small regions of about 0.5 μm^2 may have AcChR site densities approaching several thousand sites per μm^2. Because the emulsion over such small regions saturates with relatively few developed grains, accurate determination of site density in such small regions is not possible on the light autoradiographic level. However, because the high-density patches occurred rarely and occupied a relatively small fraction of the total area in the randomly sampled myotubes, there was no significant effect if these patches were omitted from the calculated average AcChR site density.

The largest increase in receptor site density that we have observed was on myotubes near the spinal explants. However, the increase was not limited to the region immediately surrounding the explant but was also found on myotubes many millimeters away from the explants (see Table 2). In fact, the site density in these more distant regions was identical to that found with the cell-free extracts. These observations strongly support the idea that a substance is diffusing from the explants and is not limited to nerve processes growing from the explants making contact with myotubes. After this study was completed, a similar effect of cord explant on acetylcholine sensitivity of chick myotubes in culture was reported by Cohen and Fisch-

Table 1. Effect of the addition of exogeneous material on the acetylcholine receptors of L_6 myotubes assessed by fluorescence after binding of rhodamine-labeled α-bungarotoxin

Material	mg protein	Patch frequency
Spinal cord extract (9)	0.762	++
Brain extract (10)	0.756	++
Dialyzed brain extract (2)	0.911	++
Liver extract (2)	0.785	−
Amniotic fluid (4)	1.431	−
12-day spinal cord and brain extract (4)	0.959	−
Adult brain extract (1)	0.941	+
Adult spinal cord extract (1)	0.954	+
Spinal explant medium (1)	—	−
Spinal explant (6)	—	+++
Primary muscle medium (4)	—	−

Numbers in parentheses are the numbers of experiments done with each material. Column heading "mg protein" represents the total protein added over 3 days. Observations of patch frequency were only semiquantitative and results are represented by symbols as follows: +++ = 10^2–10^3 patches per mm^2 of tissue culture dish; ++ = 10^1–10^2 patches per mm^2; + = 1–10 patches per mm^2; − represents less than 1 patch per mm^2.

Table 2. Effect of the addition of neuronal material on the acetylcholine receptors of L_6 myotubes assessed by quantitative light autoradiography after binding of ^{125}I-labeled α-bungarotoxin

Experimental condition	Average sites/μm^2	Random myotubes	High–low sites/μm^2
L_6 control	20 ± 3 (n = 935)	15	58–4
Spinal explant, 2 mm	127 ± 12 (n = 2664)	15	247–37
Spinal explant, 5 mm	76 ± 10 (n = 1831)	13	
18-day brain extract			
1.8–2.3 mg	79 ± 13 (n = 1010)	6	175–34
1.4 mg	77 ± 11 (n = 874)	8	208–30
0.2–0.9 mg	46 ± 5 (n = 820)	9	130–14
18-day spinal cord extract, 1.0 mg	61 ± 6 (n = 455)	3	119–28
Primary rat control	204 ± 34 (n = 785)	5	290–65

Average site densities were obtained from 3–15 randomly selected myotubes (see text) and are given ± standard error of the mean. The number of total grains (n) used in the calculation is indicated in parentheses. The area tabulated per myotube varied from 800 to 2400 μm^2. Because the autoradiographic sampling error is $\sqrt{n}$, it was well below 10% in all cases. Background BTX site density on mononucleated cells was 11 ± 2 sites per μm^2 and was substracted from each of the above values. The mg values are the mg of protein added over a 3-day period. High–low sites per μm^2 refers to averaged site densities on two selected myotubes. These were selected after scanning the cells in the dishes to locate one myotube having a high overall label along its full length, and a second having a low overall label. These two values were not included in the average values obtained from randomly selected myotubes and were chosen to give an impression of the full range of site densities for different myotubes in all the dishes used for each treatment. Note that the randomly selected tubes invariably fell within this range. The values for the spinal cord explants were obtained from myotubes within 2 mm of the explant and at distances greater than 5 mm; otherwise the myotubes were selected randomly. The values for the primary rat myotubes agree with those previously reported (8). Note primary rat myotubes were not treated with nerve factors.

bach (14). The questions of whether nerve muscle contact was necessary or if the effect was specific to motor neurons was not resolved in that study.

We performed three types of experiments which suggest that the substance in the extracts causing the effects on the receptor site density is a macromolecule and is probably a protein(s). First, dialysis sometimes reduced the activity, but significant activity always remained after extensive dialysis. Second, filtration of the extract through Sephadex G-100 showed that the activity filtered as one peak with a molecular weight of about 100,000. A precise molecular weight estimate will require additional work. Third, trypsinization (1 mg/ml) of the extract for 60 min at 37° abolished the activity. (The trypsin was inactivated by diisopropyl fluorophosphate, which was then removed by dialysis prior to adding the extract to the culture.[¶]) These observations suggest that the activity resides in one or more protein or complex proteins of roughly the same molecular weight.

[¶] Three types of control samples were tested simultaneously with the trypsin treatment. One sample was treated in an identical way as the trypsinized one except no trypsin was added. A second one was left at 4° while the trypsinization was done at 37°, then the 4° sample was treated with diisopropyl fluorophosphate and dialyzed with the other samples. A third sample was simply left at 4° while the others were being dialyzed at 4°. All these control samples showed positive results when tested for the formation of AcChR patches. A sample of trypsin treated with diisopropyl fluorophosphate and then dialyzed had no effect.

The effect of the nerve extract on overall protein synthesis was determined, and approximately a 20% increase was shown by both sodium dodecyl sulfate extraction of cells followed by Lowry protein determination and [^{3}H]leucine incorporation under conditions in which there was no large change in the number of cells. This effect is similar to that reported by Oh (15) on primary muscle cultures.

DISCUSSION

We have shown that substances released from embryonic nervous system explants and contained in nervous system cell-free extracts can alter the AcChR in cultured L_6 cells in two important ways: (*i*) there is an increase in average AcChR site density; and (*ii*) high-density patches of receptors are formed. These results clearly demonstrate the presence of a diffusible factor that is capable of causing these changes in AcChR number and distribution independently of innervation or nerve contact.

Several studies have recently used nerve extracts in an effort to identify neurotrophic factors (15–19). These studies have found that proteins are the primary active component in the extracts, but the molecular weights of the proteins have ranged between 10,000 and 300,000. Much more work will be necessary to characterize these proteins in order to determine their relationship to one another. In addition to these proteins, it has also been suggested that small molecules such as cyclic AMP may also be involved (20, 21). Ravdin (22), however, has shown that neither derivatives of cyclic AMP nor agents that increase the cyclic AMP levels cause the formation of high-density patches of receptors in L_6 cells. Therefore, in the L_6 cells, cyclic AMP by itself is not sufficient to cause this change in AcChR distribution.

Because untreated L_6 myotubes have a very low and random AcChR distribution, the treatment of these cells with central nervous system explants or extracts causes them to resemble primary rat muscle cultures at least with respect to the presence of AcChR patches. The differences between the untreated L_6 cells and primary muscle cells may, therefore, be due to the exposure of the primary cells to neurotrophic factors *in situ*. Any such neural influence may have disappeared in L_6 cells because of the long period of time they have existed in culture (5). On the other hand, the presence of other cells such as fibroblasts in primary culture cannot be excluded as a source of the stimulatory factor. It remains uncertain, however, what relationship these observations have to synaptogenesis *in situ*, because the role, if any, of the patches of AcChR in synaptogenesis is unknown.

Anderson *et al.* (23, 24) have shown a redistribution of existing AcChR to form high-density patches in the presence of dissociated neural tube cells and argue that this redistribution may be the primary mechanism in the formation of neuromuscular junctions. A preliminary report by Christian *et al.* (25) also claims an increase in AcChR patches but no increase in AcChR site density on primary mouse myotubes exposed to NG108-15 hybrid cells. In the present study using L_6 cells we found that nerve factors induce both an increase in average AcChR site density and an increase in the number of high-density patches. This suggests that redistribution and increased overall numbers of AcChRs may be independently controlled by nerve factors.

It is possible that the increase in AcChR site density is more pronounced when the initial level of receptor is low, as it is in the L_6 clones used in this study. The mechanism and control of this process remain to be investigated. The availability of the L_6 test system and the apparent dose-dependent quantitative

response of its AcChR site density to some trophic factor provides a powerful bioassay to test for the presence of these factors in cell-free extracts of nervous and other embryonic tissue.

We wish to thank Professors W. W. Webb and E. L. Elson for the use of their laboratory facilities, and S. Nichols for her aid in maintaining the L_6 cells. We acknowledge the following sources of financial support: National Institutes of Health Grants NS-10638 (T.R.P.), GM 10422 (M.M.S.), and GM 21661 (W. W. Webb and E. L. Elson); National Science Foundation Condensed Matter Grant DMR 75-04509 (W. W. Webb); and National Institutes of Health Postdoctoral Fellowship IF 32NS05621 (D.A.).

1. Nelson, P. G. (1975) *Physiol. Rev.* **55**, 1–61.
2. Gutman, E. (1976) *Annu. Rev. Physiol.* **38**, 177–216.
3. Sytkowski, A. J., Vogel, Z. & Nirenberg, M. W. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 270–274.
4. Fischbach, G. D. & Cohen, S. A. (1973) *Dev. Biol.*, **31**, 147–162.
5. Yaffe, D. (1968) *Proc. Natl. Acad. Sci. USA* **61**, 477–483.
6. Kidokoro, Y. & Heinemann, S. (1974) *Nature* **252**, 593–594.
7. Land, B. R., Podleski, T. R., Salpeter, E. E. & Salpeter, M. M. (1977) *J. Physiol. (London)* **221**, 743–754.
8. Patrick, J., Heineman, S. F., Linstrom, J., Schubert, D. & Steinbach, J. H. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2762–2766.
9. Steinbach, J. H., Harris, A. J., Patrick, J., Schubert, D. & Heineman, S. (1973) *J. Gen. Physiol.* **62**, 255–274.
10. Axelrod, D., Ravdin, P., Koppel, D. E., Schlessinger, J., Webb, W. W., Elson, E. L. & Podleski, T. R. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4594–4598.
11. Ravdin, P. & Axelrod, D. (1977) *Anal. Biochem.* **80**, 585–592. See also erratum (1977) **83**, 336.
12. Fertuck, H. C. & Salpeter, M. M. (1976) *J. Cell Biol.* **69**, 144–158.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
14. Cohen, S. A. & Fischbach, G. D. (1977) *Dev. Biol.* **59**, 24–38.
15. Oh, T. H. (1976) *Exp. Neurol.* **50**, 376–386.
16. Oh, T. H., Johnson, D. D. & Kim, S. U. (1972) *Science* **178**, 1298–1300.
17. Oh, T. H. (1975) *Exp. Neurol.* **46**, 432–438.
18. Singer, M., Maier, C. E. & McNutt, W. S. (1976) *J. Exp. Zool.* **196**, 131–150.
19. Festoff, B. W. & Israel, S. (1976) *Neurosciences Abstracts* **2**, 1040.
20. Lentz, T. L. (1974) *Exp. Neurol.* **45**, 520–526.
21. Carlsen, R. C. (1975) *J. Physiol. (London)* **247**, 343.
22. Ravdin, P. (1977) Dissertation (Cornell University, Ithaca, NY).
23. Anderson, M. J., Cohen, M. W. & Zorychta, E. (1977) *J. Physiol. (London)* **268**, 731–756.
24. Anderson, J. J., & Cohen, M. W. (1977) *J. Physiol. (London)* **268**, 757–773.
25. Christian, C. N., Daniels, M., Sugiyama, H., Vogel, Z. & Nelson, P. G. (1977) *Neurosciences Abstracts* **3**, 533.