

Synapse and acetylcholine receptor synthesis by neurons dissociated from retina

(¹²⁵I-labeled α -bungarotoxin/autoradiography/cultured embryonic cell aggregates/electron microscopy)

ZVI VOGEL*, MATHEW P. DANIELS, AND MARSHALL NIRENBERG

Laboratory of Biochemical Genetics, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

Contributed by Marshall Nirenberg, May 10, 1976

ABSTRACT Neurons dissociated from chick embryo retina and cultured form more than 1×10^8 synapses per mg of protein. At least three types of synapses are synthesized which resemble those of the intact retina. In addition, two populations of neurons were found, those with nicotinic acetylcholine receptors and those without the receptors.

Sheffield and Moscona (1) and Stefanelli *et al.* (2) have shown that cultured neurons dissociated from chick embryo retina form synapses *in vitro*. Since only five types of neurons are present in the vertebrate retina and both the structure and function of the neurons and synaptic circuits of the retina have been studied extensively (3-5), chick embryo retina would appear to be an excellent cell system for studying the formation of synapses.

Avian retina and retina from several mammalian species contain relatively high concentrations of nicotinic acetylcholine (ACh) receptors, that are primarily associated with the synaptic layers of the retina (6, 7). Acetylcholine receptor synthesis is an early event in chick embryo retina when compared with the appearance of synaptic connections. In this report, the formation of synapses by cultured neurons that were dissociated from chick embryo retina is correlated with the synthesis of nicotinic ACh receptors.

METHODS

Retina Dissociation. Eight-day-old chick embryo retinas were dissociated into single cells essentially as described by Sheffield and Moscona (1). Retinas were dissected in Eagle's basal medium (BME, Microbiological Associates), washed, cut into pieces, and incubated for 15 min at 37° in Tyrode's solution without Ca⁺⁺ or Mg⁺⁺ in an atmosphere of 5% CO₂-95% air. Trypsin (crystallized three times) and DNase I (crystallized, Worthington Cat. no. 2058) were added so that the final concentrations were 2.5 and 0.05 mg of protein per ml, respectively (about 0.3 ml per retina). The tissue was incubated for an additional 15 min, then 0.5 ml of cold growth medium (80% BME with 2 mM glutamine and 20% fetal bovine serum) was added per retina and the partially disrupted tissue was sedimented by low-speed centrifugation. The pellet was mixed with 0.3 ml of culture medium per retina and dispersed into single cells by pipetting in and out of a pasteur pipette with a fire-polished tip. Each retina yielded 30 to 40 $\times 10^6$ cells. At least 95% of the cells were single cells; a few clusters comprised of two to three cells also were present. Cell viability, determined by dye exclusion, was >95%.

Monolayer Cultures. A cell suspension (9×10^6 cells in 1.5 ml of growth medium) was added to each 35 mm collagen-

coated tissue culture petri dish (Falcon Plastics). Cultures were incubated at 37° in a humidified atmosphere of 95% air-5% CO₂. The culture medium was changed every other day.

Aggregate Cultures. A cell suspension (9×10^6 cells in 1.5 ml of growth medium) was added to each 35 mm petri dish (bacteriological, rather than tissue culture grade, Cat. no. 1008, Falcon Plastics) and the dishes were swirled on a gyratory shaker (Bellco) with an excursion of approximately 2.6 cm at 80 rpm in a 37° incubator with a humidified atmosphere of 95% air-5% CO₂. The cells adhere to one another and form aggregates. Two-thirds of the culture medium (1.0 ml) was changed every other day.

Assay of Homogenates for ¹²⁵I-Labeled α -Bungarotoxin Binding. Aggregates from one to three dishes were collected by gentle centrifugation, washed three times with Dulbecco's phosphate buffered saline (8), and homogenized in 0.05 M Tris-HCl, pH 7.4. Monolayer cultures of retina cells were washed with Dulbecco's phosphate-buffered saline, scraped from the dish in that buffer, sedimented in a Duall ground glass conical homogenizer (Kontes), and homogenized in 0.05 M Tris-HCl, pH 7.4. The homogenates were incubated with 10 nM ¹²⁵I diiodo-labeled α -bungarotoxin, henceforth termed ¹²⁵I-labeled α BT, for 30 min at 37° and the amount of ¹²⁵I-labeled α BT bound was determined by filtration through a cellulose acetate filter (EGWP; Millipore Co.) 25 mm in diameter with a 0.2- μ m pore size (7). ¹²⁵I-labeled α BT was prepared as described previously (9). Protein was determined by a modification of the method of Lowry *et al.* (10).

Autoradiography of Cultured Cells. Cultures were incubated with 10 nM ¹²⁵I-labeled α BT in growth medium for 1 hr and then cooled on ice. Monolayer cultures were washed five times with cold growth medium (2 ml each wash) and twice with cold growth medium without serum. The total wash time was 15 min. Aggregates were transferred to conical siliconized (Siliclad) glass tubes and washed as first described *except* that aggregates were recovered by centrifugation. All cultured material was fixed for 1 hr or longer with cold 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, containing 2 mM CaCl₂, and rinsed six times for 30 min to 12 hr (total) with 0.1 M sodium cacodylate, pH 7.4 containing 2 mM CaCl₂. Monolayer culture dishes then were washed briefly with water, excess fluid was removed, and the cultures were coated with Kodak NTB-2 emulsion. Autoradiographs were exposed and developed as previously described (11). Aggregates fixed and washed as described above were dehydrated, cleared, and embedded in paraffin by standard methods. Sections 6 μ m thick were mounted on glass slides, deparaffinized, subjected to autoradiography, and stained with toluidine blue as described previously (7). Some cultured aggregates were dehydrated in ethanol and propylene oxide and embedded in Epon. Sections 0.5 μ m thick were mounted on glass slides, subjected to autoradiography, and stained with toluidine blue.

Electron Microscopy. Aggregates fixed and washed as de-

Abbreviations: α -BT, α -bungarotoxin; ¹²⁵I-labeled α BT, α -bungarotoxin labeled with 2 atoms of ¹²⁵I per toxin molecule; ACh, acetylcholine.

* Present address: Neurobiology Unit, The Weizmann Institute of Science, Rehovot, Israel.

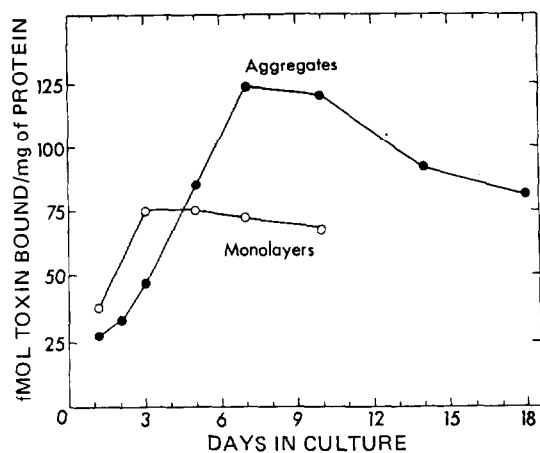


FIG. 1. Binding of ¹²⁵I-labeled αBT to cultured cells dissociated from retina and grown *in vitro* for different periods. Cells from 8-day-old chick embryo retina were dissociated and cultured as cell aggregates in rotating petri dishes or as monolayers in stationary petri dishes. Each petri dish was inoculated with 9×10^6 cells. Cells were harvested, homogenized, and assayed for binding of ¹²⁵I-labeled αBT as described under *Methods*. Symbols represent the following: fmol of toxin bound per mg of protein with cell aggregates (●) and with stationary cell monolayer cultures (○).

scribed above, were postfixed for 1 hr in 1% OsO₄ in 0.1 M sodium cacodylate, pH 7.4, containing 2 mM CaCl₂ and dehydrated and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate.

RESULTS

Activity and distribution of ACh receptors

Cells dissociated from chick embryo retina on the 8th embryonic day, were cultured for 1–10 days, either in stationary or rotating petri dishes to obtain cell monolayers or aggregates, respectively. Homogenates were prepared at different times as indicated in Fig. 1 and the amount of ¹²⁵I-labeled αBT bound specifically was determined. With cell aggregates, specific binding of ¹²⁵I-labeled αBT increased 5-fold between the 1st

and 7th day *in vitro* and the number of αBT binding sites in aggregates cultured for 5 days was equal to that of the intact retina of the same age (13th embryonic day). The maximum concentration of receptors was attained between 7 and 10 days *in vitro* and then gradually declined. The decrease in receptors may result from cell death, for necrotic areas were found in some of the larger aggregates. With stationary cell monolayer cultures, receptor concentration increased 2-fold between the 1st and 3rd culture days and did not change thereafter. The maximum concentrations of αBT binding sites found with cell aggregates, monolayers, and intact retina of newly hatched chicks were 125, 75, and 400 fmol, respectively, of toxin bound specifically per of mg of protein (30 min incubation with 10 nM ¹²⁵I-labeled αBT at 37°).

Autoradiographs of sections of cell aggregates cultured for 1–7 days and then incubated with ¹²⁵I-labeled αBT are shown in Fig. 2. As shown in panel A, the cell bodies and ACh receptors within the 1-day-old aggregate are distributed more uniformly than in older aggregates (panels B and C), but small areas with higher levels of ¹²⁵I-labeled αBT binding are present after 1 day of incubation. Larger aggregates are present after 7 days of culture (panels B and C) and cell bodies and processes (neurites) have sorted out into discrete regions which are equivalent to the layers of cell bodies and processes in intact retina. The neurite rich regions are heavily labeled with ¹²⁵I-labeled αBT. Little binding of ¹²⁵I-labeled αBT was observed in the presence of *d*-tubocurarine (Fig. 2C) which competes with αBT for binding sites on the nicotinic ACh receptor. Previous studies with *d*-tubocurarine and other ligands of the nicotinic ACh receptor have shown that the specificity of αBT binding sites of chick embryo retina for various ligands is similar to that of the nicotinic ACh receptor (7). The sorting out of neurites and ACh receptors from cell bodies was observed consistently in aggregates cultured for 3 or more days.

Photomicrographs at higher magnification of 0.5 μm Epon sections of retina cell aggregates cultured for 7 days are shown in Fig. 3. The use of Epon sections results in improved autoradiographic resolution as well as superior cell structure as compared to the paraffin sections. In Fig. 3A a section which was not subjected to autoradiography is shown in order to show cell

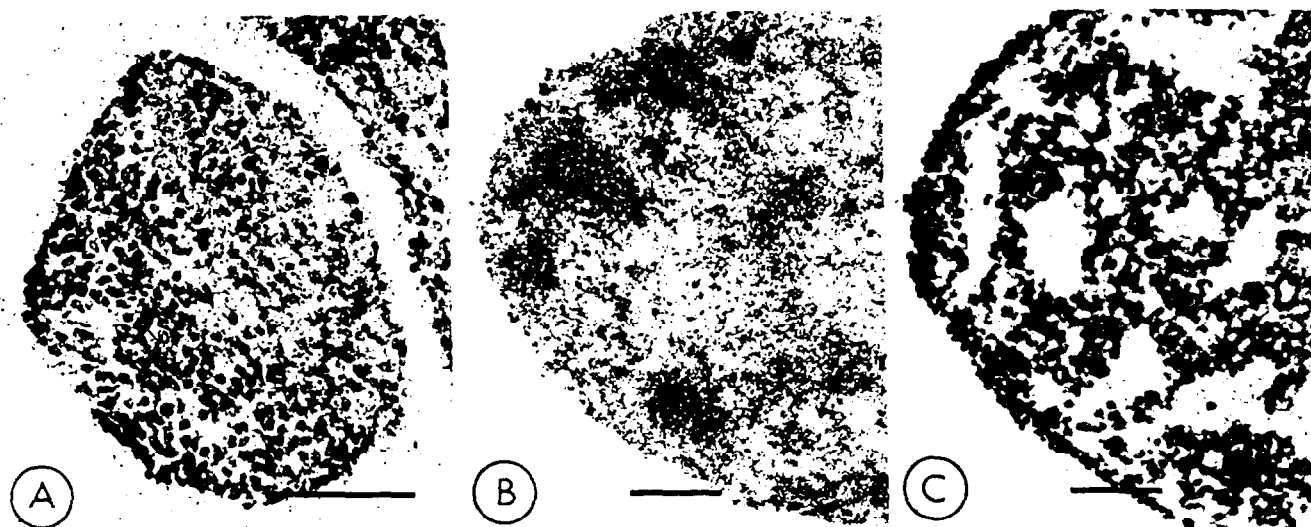


FIG. 2. Autoradiographs of 6 μm thick sections of retina cell aggregates cultured for 1 or 7 days, to illustrate the progressive sorting out of neurites with nicotinic ACh receptors from cell bodies. The cell aggregates were incubated with ¹²⁵I-labeled αBT (300 Ci/mmol of toxin), sectioned, subjected to autoradiography for 36 days, and stained with toluidine blue. A, Cell aggregates cultured for 1 day. B, A cell aggregate cultured for 7 days. C, A cell aggregate cultured for 7 days and incubated for 10 min with 0.5 mM *d*-tubocurarine before the addition of ¹²⁵I-labeled αBT. The bars represent 50 μm. The areas packed with neurites appear black in panel B due to the relatively high concentration of silver grains resulting from ¹²⁵I-labeled αBT bound to receptors on neurites. In panel C these regions appear to be relatively devoid of silver grains due to inhibition of αBT binding by *d*-tubocurarine.

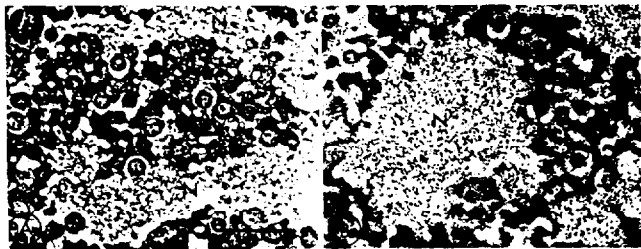


FIG. 3. Distribution of bound ^{125}I -labeled αBT in neurite-rich regions compared with cell body regions. Phase contrast views of toluidine blue stained $0.5\ \mu\text{m}$ thick Epon sections of retina cell aggregates cultured for 7 days. A (left), A section not subjected to autoradiography. B (right) A section of an aggregate which was incubated with ^{125}I -labeled αBT ($250\ \text{Ci/mmol}$ of toxin) and subjected to autoradiography for 50 days. The microscope was focused on the silver grains. The letter N corresponds to areas packed with neurites. The bar represents $25\ \mu\text{m}$.

body and neurite structure more clearly. A similar section subjected to autoradiography is shown in Fig. 3B. Most of the silver grains are associated with neurites rather than cell bodies.

Ultrastructure of the neurite regions

Aggregates of cells dissociated from 8-day-old chick embryo retinas were fixed for electron microscopy after 1–21 days of culture. The retinal neurons of 1-day-old aggregates had extended many neurites (Fig. 4A) which were loosely packed in the cell aggregates. No synapses were seen at 1 or 2 days although a few synaptic lamellae (ribbons) and small electron lucid vesicles were present. However, other types of inter-

cellular junctions such as macula adhaerens diminuta and zonula adhaerens were present, as reported by Sheffield and Moscona (1). At 5 days the neurite regions were larger and neurites were packed closer together. A few, immature synaptic connections were seen at this time. By 7 days (Fig. 4B), the intercellular gap between closely packed neurites was reduced to a width of about $200\ \text{\AA}$ at most points. Synapses now were very abundant; the average thin section of neurites sorted out from cell bodies contained approximately 30 synapses per $100\ \mu\text{m}^2$. The maximum number of synapses ($38\ \text{synapses per } 100\ \mu\text{m}^2$) was achieved by the 14th day *in vitro*. Similar counts with electron micrographs of inner synaptic layer of adult chick retina revealed about 50 synapses per $100\ \mu\text{m}^2$. Assuming that the diameter of the average synapse is $1\ \mu\text{m}$, the number of synapses found in a thin section $0.05\ \mu\text{m}$ in thickness, correspond to a tissue section with a maximum thickness of $2\ \mu\text{m}$. Thus, a thin section $100\ \mu\text{m}^2$ is equivalent to $200\ \mu\text{m}^3$ of tissue with respect to the detection of synapses. Assuming that neurite regions comprise about 40% of the cell aggregates, and protein about 10% of the cell aggregate wet weight, the number of synapses formed by cultured retina cells is $1.5\ \text{to } 2 \times 10^9/\text{mg}$ of protein; this is a remarkably high value and is in the range of that found in the intact retina (12). The minimum number of synapses formed *in vitro* is, by conservative estimate, $>1 \times 10^8/\text{mg}$ of protein.

Many synapses in 14- to 21-day-old aggregates appeared more mature than those of 7-day-old aggregates. For example, the submembrane densities were more extensive and the electron lucid (synaptic) vesicles more closely packed. Between 14 and 21 days, necrotic areas appeared in the larger aggregates,

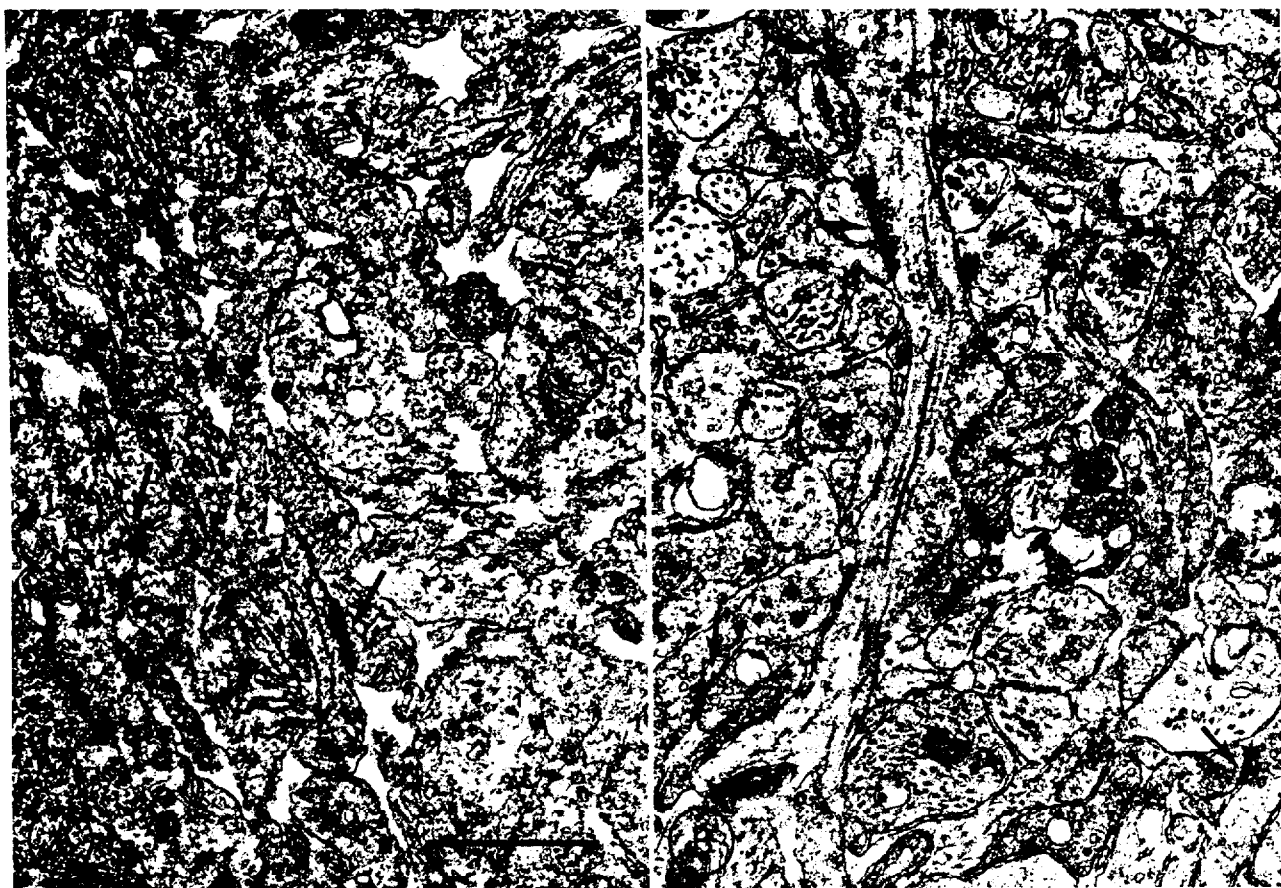


FIG. 4. Neurite-rich regions of aggregates of 8-day-old chick embryo retina cells. A (left), Cells cultured 1 day. The neurites are loosely packed. The arrows refer to macula adhaerens junctions. Synaptic junctions have not yet formed. B (right), Cells cultured 7 days. The neurites are closely packed and many synapses (arrows) are present. Bar represents $1\ \mu\text{m}$.



FIG. 5. Synapses in neurite-rich regions of aggregates of 8-day-old chick embryo retina cells. A (upper panels), Cultured 18 days. Several synapses of the amacrine-amacrine type are present. Neurite labeled (2) appears to be postsynaptic to neurites (1) and (3) whereas (3) is postsynaptic to (4). Note presynaptic tufts of electron dense material (arrows). B (middle panel), Cultured 14 days. A bipolar ribbon synapse with presumed ganglion and/or amacrine neurons is present. Note the synaptic ribbon, dense cap, and synaptic vesicles in the bipolar axon terminal and the densities under the postsynaptic membranes. A small symmetrical junction also is present (arrow). C (lower panel) Cultured 10 days. Two photoreceptor cell processes in synaptic contact with several neurite endings. The photoreceptor processes contain many synaptic ribbons, electron lucid vesicles, and a few dense-core vesicles (d). Regions of presumed synaptic contact, with submembrane densities are indicated (arrows). The alignment and opposition of synaptic ribbons of two different photoreceptor cell processes seen here was unusual, and was not observed in the intact chicken retina. Bar represents 0.5 μ m.

and some neurites appeared to be replaced by Müller cell processes.

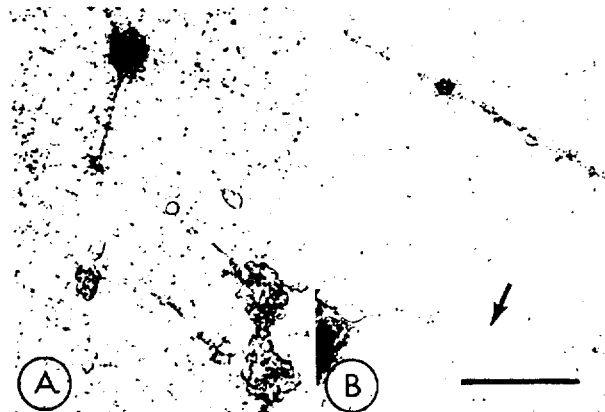


FIG. 6. Bright-field views of monolayer cultures of chick embryo retina cells incubated with 125 I-labeled α BT and subjected to autoradiography. Labeled and unlabeled cells and cell processes can be seen. A, Retina cells (9×10^6 cells from 8-day-old embryonic retina inoculated per 35-mm petri dish), cultured for 10 days, incubated with 125 I-labeled α BT (300 Ci/mmol of toxin), and subjected to autoradiography for 36 days. The neurons are attached to a confluent monolayer of cells resembling fibroblasts which are not labeled appreciably and thus are not seen clearly. B, Retina cells (7×10^5 cells inoculated per 35-mm petri dish) cultured for 7 days, incubated with 125 I-labeled α BT (410 Ci/mmol of toxin) and subjected to autoradiography for 30 days. The arrow indicates an unlabeled cell process. The bar represents 50 μ m.

Three types of synapses were identified in the neurite regions. Conventional synapses (Fig. 5A) which resemble amacrine-amacrine synapses of the intact retina and which are similar to those reported by Stefanelli *et al.* (2) were observed most frequently. At these synapses, synaptic vesicles were abundant on one side of the cleft, but were scattered or absent on the other side; electron dense material was present under pre- and post-synaptic membranes; and, in some cases, one or more tufts of electron dense material projected intracellularly from the presynaptic density. Single neurites frequently formed two or more conventional synapses; serial synapses also were observed.

A second type of synapse (Fig. 5B) resembled the synapse between a bipolar neuron and two postsynaptic processes of amacrine and/or ganglion neurons. The presynaptic ending contained a short synaptic ribbon surrounded by a cluster of vesicles close to a convex electron-dense cap at the tip of the ending. The postsynaptic endings had electron-dense material under the plasma membrane in the vicinity of the cap. In some cases, one or both postsynaptic endings contained synaptic vesicles as with amacrine neurons in the intact retina.

A third type of synapse (Fig. 5C), resembled the synapse of a photoreceptor cell with neurites of horizontal and/or bipolar neurons. The large presynaptic processes contained synaptic ribbons, many electron-lucid synaptic vesicles, and a few dense core vesicles. Stefanelli *et al.* (2) described processes of this type in retina cell aggregates and Sheffield and Moscona (1) identified photoreceptor cell processes by the presence of paraboloid and ellipsoid bodies in the cell body. We also identified photoreceptor cell processes in this manner. Neurite endings with submembrane electron dense material were found in the invaginations of photoreceptor processes, sometimes near synaptic ribbons. Photoreceptor ribbon synapses with one or two postsynaptic processes were found more frequently than triad synapses with three processes. Perhaps these configurations represent developmental stages in the assembly of triad synapses.

Cell aggregates were cultured for 1–21 days in the presence of 2 μ M α BT and then were fixed for electron microscopy.

α -Bungarotoxin did not inhibit the formation of the three types of synapses described above and had no obvious effect upon the abundance of synapses or their morphology. These results suggest that activation of nicotinic ACh receptors by acetylcholine is not required for the formation of at least three kinds of synapses.

Photomicrographs of stationary monolayer cultures of retina cells which were incubated with labeled α BT and subjected to autoradiography are shown in Fig. 6. Small clusters of cells which were connected to other islands of cells by long neurites often were seen on top of a monolayer of fibroblast-like cells. Some cell clusters were heavily labeled with ^{125}I -labeled α BT; others were labeled lightly or not at all. Thus, two types of cells with neuronal morphology were found: cells which bind ^{125}I -labeled α BT (usually on both cell bodies and processes) and cells which do not bind toxin.

DISCUSSION

The results show that cultured neurons dissociated from chick embryo retina synthesize nicotinic ACh receptors and extend long neurites which together sort out from cell bodies. We estimate that cultured cells form approximately 1.5×10^9 synapses per mg of protein. By conservative estimate, the minimum number of synapses formed *in vitro* would appear to be $>1 \times 10^8$ /mg of protein.

At least three types of synapses are formed *in vitro* which resemble those of the intact avian retina: bipolar ribbon synapses, photoreceptor ribbon synapses, and amacrine conventional synapses. Serial synapses and reciprocal synapses also were found which closely resemble those of amacrine neurons in the intact retina. Additional synapse subclasses were found but neuron identification was uncertain. For example, bipolar ribbon synapses were observed with different combinations of postsynaptic neurons, some with, and some without synaptic vesicles, tentatively identified as amacrine neurons and ganglion neurons, respectively.

Synapses first appear in the intact retina on the 13th embryonic day (13–15). Most of the synapses in cell aggregates formed from cells that were dissociated on the 8th embryonic day, appear between the 5th and 7th day *in vitro*, which corresponds to the 13th and 15th day *in ovo*.

Autoradiography of monolayer cells which had been incubated with ^{125}I -labeled α BT revealed two populations of neurons; those with nicotinic ACh receptors on cell bodies and processes which comprised about 20% (the range is 15–40%) of the cell population, and neurons without these receptors. Culturing monolayers of retina cells for 5–6 days in the presence of either acetylcholine (with or without eserine), carbamylcholine, or *d*-tubocurarine had little or no effect on the concentration of nicotinic ACh receptors. Thus, as reported previously for striated muscle (16, 17), ligands of the nicotinic ACh

receptor are neither required nor inhibit receptor synthesis. Cells cultured in the presence of α BT for up to 21 days were not noticeably affected with respect to the number or the kinds of synapses synthesized. Thus, activation of nicotinic ACh receptors is not required for the synthesis *in vitro* of many synapses, but the results do not rule out the possibility that α BT inhibits the formation of certain synapses. α -Bungarotoxin reportedly inhibits the normal development of the neuromuscular synapse *in vivo* (18).

Most of the nicotinic ACh receptors were associated with neurites rather than cell bodies, both in cell aggregates and in the intact retina. The sorting out of neurites and the synthesis and apparent segregation of ACh receptors, thus are early events which precede synapse formation *in vitro* and, as reported previously, *in vivo* (7). The formation of cell junctions such as the macula adhaerens and zonula adhaerens also are early events compared to the formation of synaptic connections. Thus, the developmental sequence of events leading to synaptogenesis *in vitro*, the types of synapses formed, and surprisingly, the number of synapses formed by cultured cells agrees well with the corresponding processes in the intact retina.

1. Sheffield, J. B. & Moscona, A. A. (1970) *Dev. Biol.* **23**, 36–61.
2. Stefanelli, A., Zacchei, A. M., Caravita, S., Cataldi, A. & Ieradi, L. A. (1967) *Experientia* **23**, 199–200.
3. Stell, W. K. (1972) in *Handbook of Sensory Physiology*, ed. Fourtes, M. G. F. (Springer-Verlag, Berlin, Heidelberg), Vol. 7, part 2, pp. 111–213.
4. Dowling J. E. (1970) *Invest. Ophthalmol.* **9**, 655–680.
5. Gouras, P. (1976) in *Cell Pharmacology of the Eye*, ed. Dikstein, S. (Charles C Thomas, Springfield.), in press.
6. Vogel, Z., Daniels, M. P. & Nirenberg, M. (1974) *Fed. Proc.* **33**, 1476.
7. Vogel, Z. & Nirenberg, M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1806–1810.
8. Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167–182.
9. Vogel, Z., Sytkowski, A. J. & Nirenberg, M. W. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3180–3184.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
11. Sytkowski, A. J., Vogel, Z. & Nirenberg, M. W. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 270–274.
12. Dubin, M. W. (1970) *J. Comp. Neurol.* **140**, 479–506.
13. Meller, K. (1968) in *Veröffentlichungen aus der Morphologischen Pathologie*, eds. Buchner, F. and Giese, W. (Gustav Fischer-Verlag, Stuttgart), Vol. 77, pp. 1–77.
14. Sheffield, J. B. & Fischman, D. A. (1970) *Z. Zellforsch. Mikrosk. Anat.* **104**, 405–418.
15. Hughes, W. F. & La Velle, A. (1974) *Anat. Rec.* **179**, 297–302.
16. Steinbach, J. H., Harris, A. J., Patrick, J., Schubert, D. & Heineemann, S. (1973) *J. Gen. Physiol.* **62**, 255–270.
17. Hartzell, H. C. & Fambrough, D. M. (1973) *Dev. Biol.* **30**, 153–165.
18. Giacomini, G., Filogama, G., Weber, M., Boquet, P. & Changeux, J. P. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 1708–1712.