Synapse and acetylcholine receptor synthesis by neurons dissociated from retina

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

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ABSTRACT Neurons dissociated from chick embryo retina and cultured form more than 1 X 10⁸ 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°C in Tyrode's solution without Ca²⁺ or Mg²⁺ 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 X 10⁶ 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 X 10⁶ 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°C in a humidified atmosphere of 95% air-5% CO₂. The culture medium was changed every other day.

Aggregate Cultures. A cell suspension (9 X 10⁶ 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°C 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 αBT, for 30 min at 37°C and the amount of ¹²⁵I-labeled αBT bound was determined by filtration through a cellulose acetate filter (EGWP; Millipore Co.) 25 mm in diameter with a 0.2-μm pore size (7). ¹²⁵I-labeled αBT 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 αBT 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 μm 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 μm thick were mounted on glass slides, subjected to autoradiography, and stained with toluidine blue.

Electron Microscopy. Aggregates fixed and washed as de-
Binding of $^{125}$I-labeled $\alpha$BT 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 $9 \times 10^6$ cells. Cells were harvested, homogenized, and assayed for binding of $^{125}$I-labeled $\alpha$BT 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 (●).

**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 $^{125}$I-labeled $\alpha$BT bound specifically was determined. With cell aggregates, specific binding of $^{125}$I-labeled $\alpha$BT increased 5-fold between the 1st and 7th day in vitro and the number of $\alpha$BT 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 $\alpha$BT 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 mg of protein (30 min incubation with 10 nM $^{125}$I-labeled $\alpha$BT at 37°).

Autoradiographs of sections of cell aggregates cultured for 1–7 days and then incubated with $^{125}$I-labeled $\alpha$BT 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 $^{125}$I-labeled $\alpha$BT 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 $^{125}$I-labeled $\alpha$BT. Little binding of $^{125}$I-labeled $\alpha$BT was observed in the presence of d-tubocurarine (Fig. 2C) which competes with $\alpha$BT 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 $\alpha$BT 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 and nuclear details. In panel B, A cell aggregate cultured for 7 days and incubated for 10 min with 0.5 mM d-tubocurarine which was stained with toluidine blue. In this panel the neurite rich areas appear black due to the relatively high concentration of silver grains resulting from $^{125}$I-labeled $\alpha$BT bound to receptors on neurites.

**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 $^{125}$I-labeled $\alpha$BT (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 $^{125}$I-labeled $\alpha$BT. 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 $^{125}$I-labeled $\alpha$BT bound to receptors on neurites. In panel C these regions appear to be relatively devoid of silver grains due to inhibition of $\alpha$BT binding by d-tubocurarine.
FIG. 3. Distribution of bound $^{125}$I-labeled $\alpha$BT in neurite-rich regions compared with cell body regions. Phase contrast views of toluidine blue stained 0.5 $\mu$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 $\alpha$BT (250 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 $\mu$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 1-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 intercellular 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 $\AA$ 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 $\mu$m$^2$. The maximum number of synapses (38 synapses per 100 $\mu$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 $\mu$m$^2$. Assuming that the diameter of the average synapse is 1 $\mu$m, the number of synapses found in a thin section 0.05 $\mu$m in thickness, correspond to a tissue section with a maximum thickness of 2 $\mu$m. Thus, a thin section 100 $\mu$m$^2$ is equivalent to 200 $\mu$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 to 2 $\times$ 10$^9$/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$^9$/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,
and some neurites appeared to be replaced by Müller cell processes.

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 postsynaptic 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 many synaptic ribbons, electron-lucid 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 μM αBT 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 αBT 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 125I-labeled αBT; others were labeled lightly or not at all. Thus, two types of cells with neuronal morphology were found: cells which bind 125I-labeled αBT (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 sort out from cell bodies. We estimate that cultured cells form approximately $1.5 \times 10^9$ synapses per mg of protein. By conservative estimate, the minimum number of synapses formed in vitro would appear to be $>1 \times 10^9$/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 6th embryonic day, appear between the 5th and 7th day in vitro, which corresponds to the 13th and 15th day in vivo.

Autoradiography of monolayer cells which had been incubated with 125I-labeled αBT 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 αBT 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 αBT inhibits the formation of certain synapses. α-Bungarotoxin reportedly inhibits the normal development of the neuromuscular synapse in vitro (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.