Proc. Natl. Acad. Sci. USA
Vol. 73, No. 6, pp. 1806–1810, June 1976
Biochemistry

Localization of acetylcholine receptors during synaptogenesis in retina

(α-bungarotoxin/neuron development/cultured neurons)

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Contributed by Marshall Nirenberg, January 9, 1976

ABSTRACT **Nicotinic acetylcholine receptors are synthesized in chick embryo retina before synapses appear. Most of the receptors are found associated with neurites in the inner synaptic layer of the retina; later in development the receptors appear in the outer synaptic layer.**

Cells dissociated from retina and cultured *in vitro* form aggregates and also sort out into regions comprised either of neurites or cell bodies. Most of the nicotinic acetylcholine receptors of aggregates are found associated with neurite regions. The receptor distribution of cultured retina cells thus resembles that of intact retina.

^{125}I-Labeled α-bungarotoxin, a specific ligand of the nicotinic acetylcholine (AcCh) receptor, was used to detect AcCh receptors in the developing chick embryo retina. This species of AcCh receptor is a synaptic marker, since the AcCh receptors of striated muscle cells (1, 2) and certain parasympathetic neurons (3) are found in abundance only at the site of the synapse. After denervation, the receptors are found over the entire surface of the cell.

Most of the recent information regarding the nicotinic AcCh receptor has been obtained with the receptors of the electric organ of fish, and striated muscle (for reviews see refs. 4–6). α-Bungarotoxin has been used to detect AcCh receptors in sympathetic ganglia (7) and brain (8–10), but the nicotinic AcCh receptors of retina have not been studied in this manner. The vertebrate retina is a model system for studies on synaptic communication, since relatively few types of neurons are present and cells dissociated from retina form synapses *in vitro* (11, 12). The retina contains acetylcholine and enzymes that catalyze the synthesis and the hydrolysis of this compound (13–17). In addition, electrophysiologic studies show that some neurons in retina respond to acetylcholine and that both nicotinic and muscarinic AcCh receptors are present in the retina of certain organisms (18–22).

In this report, the concentration of nicotinic AcCh receptors and their location in chick embryo retina are described as a function of the developmental age of the retina.

MATERIALS AND METHODS

Assay of Homogenates for ^{125}I-Labeled α-Bungarotoxin Binding. Neural retinas from white leghorn chick embryos were dissected in cold Dulbecco's phosphate-buffered saline (Gibco) and then homogenized in 0.05 M Tris·HCl, pH 7.4, in a ground glass Duall homogenizer (Kontes) using 0.25–1.25 ml of buffer per retina so that the final concentration of protein was 3–6 mg/ml. Homogenates were frozen immediately and stored at −191°; binding sites for α-bungarotoxin (αBT) were stable for at least 1 year. α-Bungarotoxin labeled with 2 atoms of ^{125}I per molecule of toxin (^{125}I-αBT) was prepared as described previously (23). Stock solutions of ^{125}I-αBT contained 0.25–0.5 μM ^{125}I-αBT; 3 mM sodium phosphate buffer, pH 7.5; 150 mM NaCl; and 2 mg of crystalline bovine serum albumin per ml. Each binding reaction mixture contained the following components in a final volume of 0.1 ml: 10 nM ^{125}I-αBT (2–4 μl of a stock solution); 0.05 M Tris·HCl, pH 7.4; 0.2 mg of serum albumin; and 0–180 μg of retina homogenate protein. Reaction mixtures were incubated for 30 min at 37°, diluted with 3 ml of solution C (0.05 M Tris·HCl, pH 7.4, and 2 mg of serum albumin per ml) at 3°, and immediately filtered (vacuum) through a wet cellulose acetate filter (Millipore, EGWP 25 mm in diameter, 0.2 μm pore size). The filter was washed four times with 3 ml portions of solution C, the filter was dissolved in 10 ml of Instabray (Yorktown Research), and radioactivity was determined. Zero time values (100–200 cpm, approximately 0.25 fmol of ^{125}I-αBT) were subtracted from the values shown. The amount of ^{125}I-αBT bound was proportional to protein concentration in the range studied (0–180 μg of protein). Protein was determined by modification of the method of Lowry *et al.* (24).

Autoradiography of ^{125}I-αBT Bound to Intact Retina. Neural retina was dissected in cold solution A [the Dulbecco-Vogt modification of Eagle's medium (Gibco Catalog no. H-21) with 20 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4, instead of $NaHCO_3$, and 2 mg of serum albumin per ml of medium]. Pieces of retina were incubated for 60 min at 37° in 0.5–1.0 ml of solution A with 10 nM ^{125}I-αBT and then were washed five times with 5 ml of cold solution A and twice with solution A without serum albumin. The tissue was fixed with 10% (vol/vol) neutral formalin or 2.5% (wt/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and embedded in paraffin. Serial sections, 8 μm thick, were mounted on glass slides, and the paraffin was removed; the slides were coated with Kodak NTB-2 emulsion diluted 1:1 with water. Slides were cooled to gel the emulsion and stored at 4° in the dark. Autoradiographs were developed for 4 min at 20° with Kodak D-19 developer diluted 1:1 with water and fixed for 5 min with Kodak F5. The slides were rinsed with water; some were stained with 0.05% toluidine blue in 0.66 M borax.

Retina Cell Cultures. Cells were dissociated from retinas of 8-day-old chick embryos and aggregated *in vitro* by a modification of the method described by Sheffield and Moscona (11). Single cells, dissociated from retina with 0.25% trypsin (three times crystallized, Worthington) and 50 μg/ml of DNase I (Worthington), were cultured in rotating flasks (80 rpm) in a 37° incubator in a humidified atmosphere of 5% CO_2–95% air. Each flask contained 9×10^6 cells and 1.5 ml of medium (80% Eagle's Basal Medium and 20% fetal bovine serum). The medium was changed every other day. After 7 days 10 nM ^{125}I-αBT was added to the aggregates in the rotating flasks. One hour later the aggregates were collected and washed four times

Abbreviations: αBT, α-bungarotoxin; ^{125}I-αBT, α-bungarotoxin labeled with 2 atoms of ^{125}I per toxin molecule; AcCh, acetylcholine.

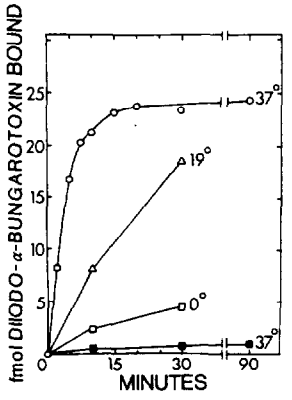

FIG. 1. The rate of ^{125}I-αBT binding. Portions of a 2-week-old chick retina homogenate (62.2 μg of protein per reaction mixture) were incubated with 1 nM ^{125}I-αBT with or without 2 μM unlabeled αBT at the temperatures and times specified.

with growth medium by centrifugation, and three times with growth medium without serum. The aggregates were fixed with glutaraldehyde, embedded in paraffin, sectioned, and subjected to autoradiography as described above.

RESULTS AND DISCUSSION

^{125}I-αBT Binding. The rate of binding of ^{125}I-αBT to receptors in a homogenate of chick retina is shown in Fig. 1. Maximum ^{125}I-αBT binding was obtained after 15 min of incubation at 37°; rates of binding were lower at 19° and 0°. Nonspecific binding of ^{125}I-αBT in the presence of a large excess of unlabeled αBT (2 μM) was relatively low.

The relation between ^{125}I-αBT concentration and the amount of labeled toxin bound is shown in Fig. 2. The amount of ^{125}I-αBT bound increased sharply as the concentration of ^{125}I-αBT was elevated from 1 to 7.5 nM. Higher concentrations of ^{125}I-αBT (10–40 nM) increased binding of labeled αBT only slightly. Little nonspecific binding of ^{125}I-αBT was observed in the presence of 2 μM unlabeled αBT. The number of specific αBT binding sites in chick retina, 2 weeks after hatching, estimated from a Scatchard plot (Fig. 2, insert), is 525 fmol/mg of protein. The (^{125}I-αBT·receptor) complex dissociates in a biphasic manner in the presence of 2 μM unlabeled αBT (50% dissociation in 30 min at 37°). Slower rates of dissociation were observed at 20° and 0° (data not shown).

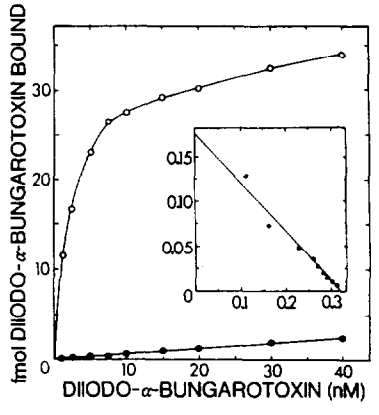

FIG. 2. Relation between ^{125}I-diiodo-αBT concentration and the amount of labeled toxin bound. Portions of a 2-week-old chick retina homogenate (62.2 μg of protein per reaction mixture) were incubated for 5 min at 20°, in the absence (○), or presence (●), of 2 μM unlabeled αBT. ^{125}I-Labeled α-bungarotoxin then was added at the concentrations specified and the reaction mixtures were incubated and assayed for bound radioactivity as described in *Materials and Methods*. A Scatchard plot of specific ^{125}I-αBT binding; i.e., binding obtained after subtracting the radioactivity bound in the presence of 2 μM unlabeled toxin, is shown in the insert; the ordinate refers to the bound/free ^{125}I-αBT ratio; the abscissa corresponds to nM toxin bound per 62.2 μg of protein.

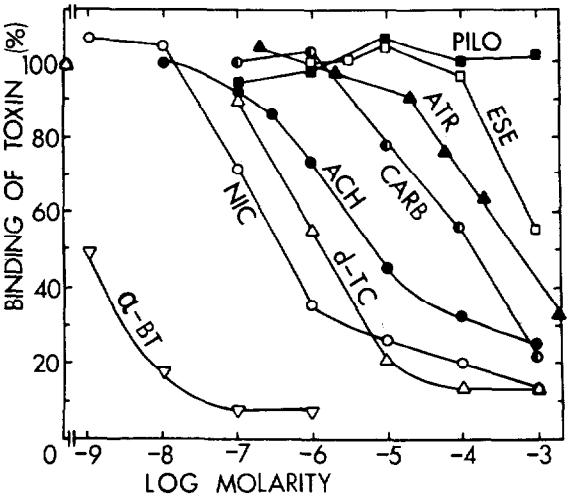

FIG. 3. Inhibitors of ^{125}I-αBT binding. Portions of a 2-week-old chick retina homogenate (50 μg of protein per 80 μl of reaction mixture) were incubated for 5 min at 20° in the presence of the compounds indicated. ^{125}I-αBT (10 nM) then was added (the final volume was 100 μl) and tubes were incubated for an additional 10 min at 20°. The final concentration of each compound is indicated on the abscissa. Tubes with acetylcholine also contained 3 μM eserine sulfate. One hundred percent on the ordinate corresponds to 6.25 fmol of ^{125}I-αBT specifically bound in the absence of inhibitor. This value was 35% of that obtained after 30 min of incubation at 37°. Abbreviations represent the following: α-BT, unlabeled α-bungarotoxin; NIC, nicotine; d-TC, *d*-tubocurarine; ACH, acetylcholine plus 3 μM eserine sulfate; CARB, carbamylcholine; ATR, atropine; ESE, eserine sulfate; PILO, pilocarpine.

Receptor Specificity. Nicotinic and muscarinic AcCh receptors can be distinguished by differences in affinity for ligands. In Fig. 3, the effects of various ligands upon ^{125}I-αBT binding are shown. Binding was inhibited 50% by 1 nM unlabeled αBT, 0.3 μM nicotine, and 1 μM *d*-tubocurarine, each compound known to have a high affinity for nicotinic AcCh receptors. Fifty percent inhibition of toxin binding was obtained with 6 μM acetylcholine (3 μM eserine sulfate also was present, a concentration without effect on αBT binding), and with 100 μM carbamylcholine. Compounds that interact preferentially with muscarinic AcCh receptors, such as pilocarpine and atropine, either did not affect ^{125}I-αBT binding or inhibited binding only at relatively high concentrations. Putative neurotransmitters such as L-glutamic acid, glycine, γ-aminobutyric acid, dopamine, and *l*-norepinephrine did not affect αBT binding at 10–1000 μM; however, serotonin stimulated αBT binding 15%. Choline (100 μM) inhibited ^{125}I-αBT binding (data not shown).

AcCh Receptors During Embryonic Development. The amount of bound ^{125}I-αBT is shown in Fig. 4 as a function of the developmental age of chick retina. Under the conditions used (10 nM ^{125}I-αBT, 30 min incubation at 37°) approximately 80% of the receptors bind αBT. Specific binding sites for αBT are present in low concentration (7.5 fmol/mg of protein) in chick retina on the 6th embryonic day. The concentration of receptors increases 10-fold between the 6th and the 13th embryonic day; however, >80% of the receptors are synthesized between the 13th and last embryonic day during the period of retina synapse formation. These results show that the receptors

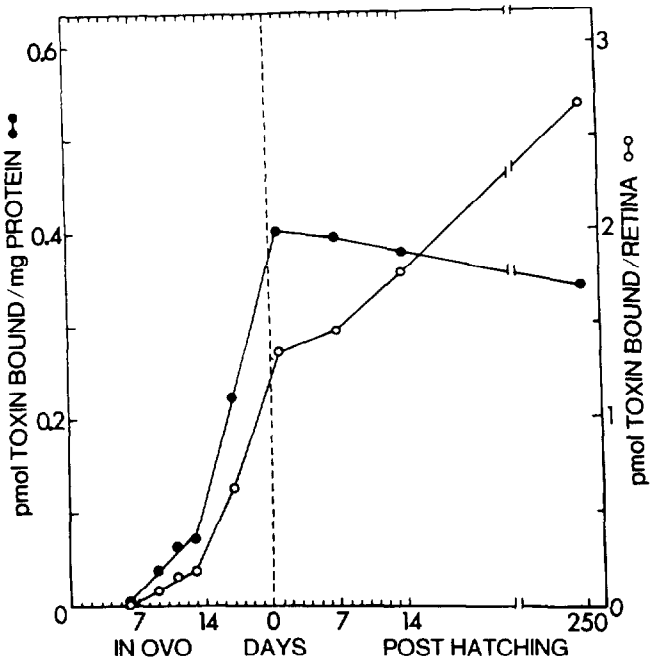

FIG. 4. Amount of ^{125}I-αBT bound as a function of the developmental age of chick retina. Filled symbols (●) represent pmol of toxin bound specifically per mg of protein; open symbols (○) represent pmol of toxin bound specifically per retina. Each point is the mean of 18 to 60 values (3 to 10 retina homogenates; six concentrations of protein were assayed from each homogenate). Specific toxin binding is shown; nonspecific binding has been subtracted.

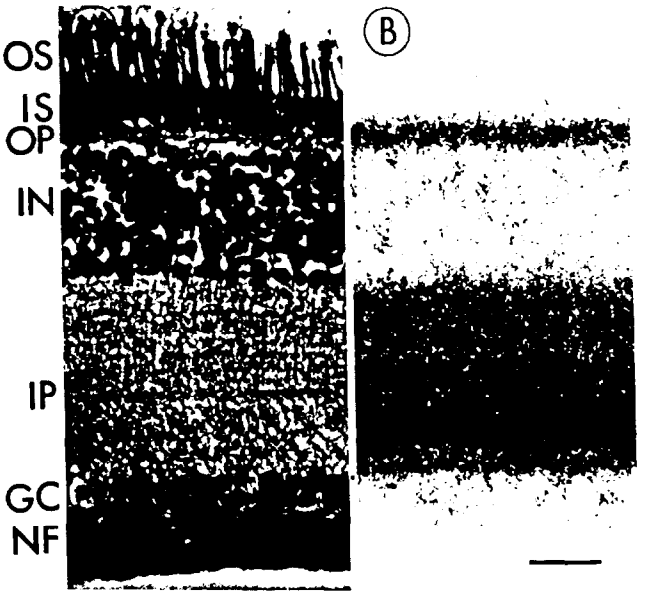

FIG. 5. Section of an 8-month-old chicken retina. (A) Toluidine-blue-stained section, not subjected to autoradiography. (B) The corresponding field in an unstained serial section, subjected to autoradiography for 110 days. The initial specific activity ^{125}I-αBT was 335 Ci/mmol of toxin. Bright field views are shown in both panels; the bar represents 25 μm. Abbreviations of retina layers are as follows: NF, nerve fiber layer, GC, ganglion cell layer, IP, inner plexiform layer (i.e., inner synaptic layer); IN, inner nuclear layer; OP, outer plexiform layer (i.e., outer synaptic layer); IS and OS, inner and outer segments of photoreceptor cell layer, respectively.

are synthesized before synapses appear and suggest that cessation of both receptor accumulation and synapse formation may be coupled. The maximum receptor concentration is attained at the time of hatching (400 fmol of ^{125}I-αBT bound per mg of protein). Almost the same receptor concentration is found in adult retina; however, the number of specific binding sites for αBT per adult retina (2700 fmol per retina) is twice that of the newly hatched chick (1350 fmol per retina). Thus, adult retina either has more synapses, more receptors per synapse, or more extrajunctional receptors than the retina of the newly hatched chick. Adult rabbit retina bound 107 fmol of ^{125}I-αBT per mg of protein under the same conditions.

Receptor Distribution in Retina. The distribution of nicotinic AcCh receptors in retina was determined by autoradiography. Adult retina was incubated with ^{125}I-αBT, fixed, sectioned, and either stained with toluidine blue or subjected to autoradiography as shown in Fig. 5A and B, respectively. Cell bodies and neurites rich in synaptic connections are found in separate layers (Fig. 5A). The upper layer consists of photoreceptor cells; next is the outer plexiform layer which consists of neurites and synapses; next is a layer of cell bodies, the inner nuclear layer; and then there is a wide layer of synaptic connections and neurites, the inner plexiform layer. The lowest layer of cell bodies is composed of ganglion neurons; the last layer consists of ganglion neuron axons.

The autoradiographs show that most of the binding sites for ^{125}I-αBT are located in the inner plexiform layer, which consists primarily of synaptic connections between processes of bipolar, amacrine, and ganglion neurons. Four horizontal bands with relatively high concentrations of silver grains can be distinguished within the inner plexiform layer. Silver grains also are found in the outer plexiform layer, which contains synapses between photoreceptor, horizontal, and bipolar neurons. Relatively few grains are associated with cell body layers, or with axons of ganglion neurons. ^{125}I-α-Bungarotoxin does not bind to pigment cells, choroid, pecten, or sclera.

Autoradiographs of sections of rat and rabbit retina are shown in Fig. 6. Again, most of the silver grains are associated with the inner plexiform layer. Some putative ganglion and amacrine neurons are labeled in rat retina (Fig. 6A); however, the outer plexiform layer of the rat is essentially unlabeled. In rabbit retina (Fig. 6B and C) silver grains are found in both the inner and outer plexiform layer. Approximately 10–20% of the rabbit ganglion cell bodies and some cell bodies in the lower portion of the inner nuclear layer, presumably amacrine neurons, are

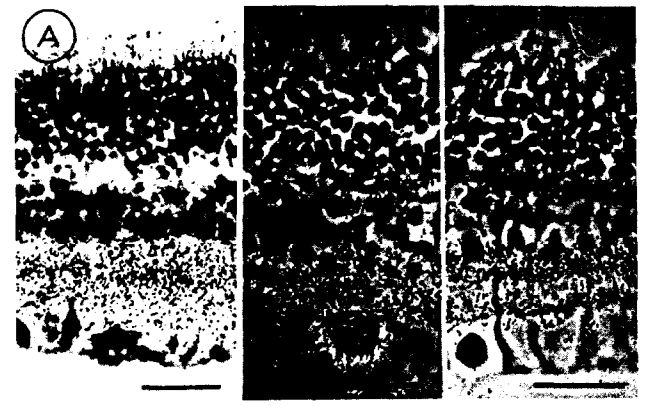

FIG. 6. Autoradiography of sections of adult rat and rabbit retina labeled with ^{125}I-αBT. (A) Bright field view of a stained section of Fisher rat retina subjected to autoradiography for 23 days. The initial specific activity of ^{125}I-αBT was 260 Ci/mmol of toxin. (B) and (C) Phase contrast views of stained sections of New Zealand white rabbit retina subjected to autoradiography for 27 days; the initial specific activity of ^{125}I-αBT was 220 Ci/mmol of toxin. The bar represents 25 μm.

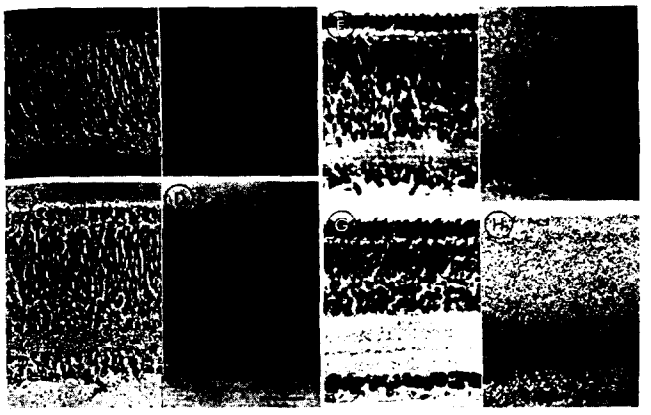

FIG. 7. Autoradiography of sections of 6- to 17-day-old chick embryo retina, labeled with ^{125}I-αBT. Retina sections were subjected to autoradiography for 29 days. The initial specific activity of ^{125}I-αBT was 485 Ci/mmol of toxin. The same magnification was used for each photomicrograph; the bars represent 25 μm. (A) Phase contrast view of a stained autoradiograph of 6-day-old embryo retina. (B) Bright field view of the area shown in panel A before staining. (C) Phase contrast view of a stained autoradiograph of 9-day-old embryo retina. (D) Bright field view of the same area shown in panel C before staining. (E) Bright field view of a stained section of 13-day-old embryo retina not subjected to autoradiography. (F) Bright field view of the corresponding area of an unstained autoradiograph of an adjacent section. (G) and (H) 17-Day-old embryo retina sections, treated as in panels E and F, respectively.

labeled. Some cells in the outer synaptic layer, thought to be horizontal neurons, also are labeled. The concentration of ganglion neurons is considerably higher in the center of the eye compared to the periphery; however, the concentration of silver grains in the inner plexiform layer does not change markedly (<2-fold).

Three types of control experiments were performed. First, incubation of chicken retina with 200 μM *d*-tubocurarine or 1 μM unlabeled αBT reduced the amount of ^{125}I-αBT bound to retina by 85% and 95%, respectively, and the few silver grains found after autoradiography were randomly distributed. Second, autoradiography of 0.5 μm thick, Epon-embedded sections of chick retina revealed the same silver grain distribution as that of 8 μm paraffin sections described above. Since Epon sections have an essentially flat surface coated with the autoradiograph emulsion, the grain distribution does not result from surface irregularities of sections after paraffin has been removed. Third, the concentration of specific ^{125}I-αBT binding sites in intact retina is approximately the same as that of homogenates prepared from sister retinas, which suggests that most binding sites in intact retina are accessible in the labeled toxin.

Embryonic chick retinas at various stages of development were labeled with ^{125}I-αBT and sections were subjected to autoradiography. Pairs of photomicrographs, one emphasizing retina structure, the other silver grain distribution, are shown in Fig. 7 for the 6th, 9th, 13th, and 17th embryonic days. The first neurons to appear are ganglion neurons which, by the 6th embryonic day, are found in a layer next to the margin of the retina (25, 26). Autoradiographs of retina on the 6th embryonic day (Figs. 7A and B) show that ^{125}I-αBT binds to ganglion neurons but not to most neuroblasts. Coulombre (25) has reported that the inner and outer plexiform layers of chick retina appear on the 8th and 9th embryonic days, respectively. ^{125}I-α-Bungarotoxin binds predominantly to the inner plexiform layer of 9-, 13-, and 17-day-old chick embryo retina. The receptors were not found in appreciable numbers in the outer synaptic layer until the 17th embryonic day.

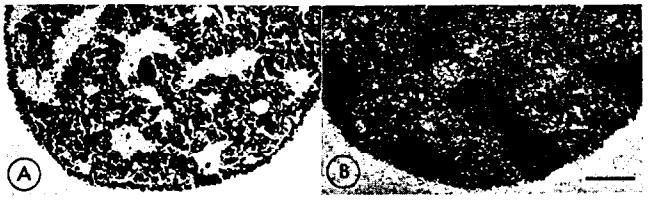

FIG. 8. Sections of an aggregate formed from dissociated 8-day-old chick embryo retina cells, cultured *in vitro* for 7 days. (A) Toluidine-blue-stained section, not subjected to autoradiography, phase contrast view. (B) Bright field view of a stained autoradiograph exposed for 36 days. The initial specific activity of ^{125}I-αBT was 300 Ci/mmol of toxin. The bar represents 50 μm.

The AcCh receptors are distributed fairly uniformly throughout the inner synaptic layer on the 9th and 11th embryonic days; by the 17th embryonic day 2 horizontal bands of receptors were observed in some autoradiographs, and in the adult, four diffuse bands of receptors can be seen within the inner synaptic layer. Acetylcholinesterase activity first appears in the 4-day-old chick embryo retina associated with ganglion neurons and later with amacrine neurons (13, 17, 18); after hatching four horizontal bands of acetylcholinesterase activity also are present in the inner synaptic layer of chick retina (17, 18).

On the 13th embryonic day retina cells with abundant AcCh receptors were found which resemble amacrine neurons. Synaptic connections first appear in chick retina on the 13th embryonic day (27–29). Thus genes for nicotinic AcCh receptors are expressed in some retina cells at least 7 days before synapses appear. Striated muscle cells also synthesize nicotinic AcCh receptors in the absence of neurons (23, 30, 31) and after denervation (2, 32, 33).

Both rod and cone photoreceptor cells of the turtle synthesize acetylcholine (16). In chick retina, nicotinic AcCh receptors are associated with dendrites of ganglion neurons and cells which resemble amacrine neurons. Our working hypothesis is that acetylcholine is a transmitter of bipolar neurons, and that some amacrine and ganglion neuron responses to bipolar neurons are mediated by nicotinic AcCh receptors. Further work also is needed to determine whether nicotinic AcCh receptors in the outer plexiform layer of chick and rabbit, but not rat, retina are synthesized by certain horizontal and/or bipolar neurons.

The relatively high concentrations of nicotinic AcCh receptors in the synaptic layers of chick retina and the low concentrations of receptors associated with cell bodies and axons of ganglion neurons suggest that nicotinic AcCh receptors of retina may be localized at certain synaptic sites much as AcCh receptors are localized at the neuromuscular synapse (1, 2) and at certain synapses of parasympathetic neurons (3). Preliminary results with an immunochemical assay for detecting nicotinic AcCh receptors at the electron microscope level (34) suggest that most of the receptors are not randomly distributed in the inner synaptic layer but instead are found in small areas which contain relatively high concentrations of receptors.

Retina Cell Cultures. Neural retina tissue from 8-day-old chick embryos was dissociated into single cells and the cells then were cultured in rotating flasks under conditions that favor aggregation as described by Sheffield and Moscona (11). A section of a cell aggregate cultured for 10 days is shown in Fig. 8A. Cell bodies and processes were found to sort out into discrete regions which resemble the layers of cell bodies and processes of the intact retina. A similar section of an aggregate which was incubated with ^{125}I-αBT and subjected to autoradiography is shown in Fig. 8B. Labeled toxin bound primarily to the neu-

rite-rich regions; considerably less toxin was associated with cell bodies. These results show that dissociated retina cells form aggregates, synthesize nicotinic AcCh receptors, and extend neurites which together with AcCh receptors sort out from cell bodies.

Cultured retina cells can be used as a model system for studies related to the process of synapse formation. At least three types of synapses form *in vitro* (11, 12), and the concentration of synaptic connections synthesized *in vitro* almost equals that found in the intact retina (35).

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