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Biochemistry

Identification of synaptic acetylcholine receptor sites in retina with peroxidase-labeled α-bungarotoxin

(neurotransmitter/central nervous system/nerve cell membrane/histochemistry/electron microscopy)

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ABSTRACT **An α-bungarotoxin-horseradish peroxidase conjugate, which binds specifically to nicotinic acetylcholine receptors, was synthesized. This conjugate was bound by 5–7% of the synapses in the inner plexiform layer of the chicken retina. Bipolar ribbon synapses as well as amacrine synapses bound the conjugate.**

The idea that certain neurotransmitter receptors are located predominantly at chemical synapses has been supported by acetylcholine (AcCh) sensitivity mapping of the skeletal muscle membrane (1) and parasympathetic neurons (2) and by electron microscopic identification of nicotinic AcCh receptor sites in the skeletal neuromuscular junction by α-bungarotoxin (αBT) binding (3–5). Radioactively labeled αBT has been used to detect nicotinic AcCh receptors in the central nervous system (6, 7) and to determine their location by light microscopic autoradiography (8–12). However, there is no evidence, either from high resolution sensitivity mapping or from electron microscopy, for the precise location of the neurotransmitter receptors on neurons in any part of the central nervous system.

There is evidence for cholinergic synaptic transmission in the vertebrate retina (for review see ref. 13; see also refs. 14–16). The synaptic layers of the retinas of several species, particularly the inner plexiform layer of the chicken retina, contain high concentrations of nicotinic AcCh receptors (9–11). We describe here the use of αBT crosslinked to horseradish peroxidase (HRP) to identify nicotinic AcCh receptor sites in the chicken retina. We present evidence for synaptic location of nicotinic AcCh receptors and a partial characterization of the synapses that contain the receptors.

METHODS

Synthesis of αBT-HRP. αBT was purified from *Bungarus multicinctus* venom (Miami Serpentarium) by the method of Mebs *et al.* (17). Monoiodinated ^{125}I-labeled αBT was synthesized as described elsewhere (18). The αBT-HRP conjugate was synthesized by a modification of the method of Avrameas and Ternynck (19). Ten milligrams of HRP (Worthington Biochemical Co., code HPOFF) was dissolved in 170 μl of 0.1 M sodium phosphate buffer, pH 6.8; 30 μl of 8% (wt/vol) aqueous purified glutaraldehyde (Polysciences) was added, and the solution was left at 20° in a brown bottle for 20 hr. A 6-ml column of Sephadex G-25 fine was prepared with 0.15 M NaCl (saline); 0.2 ml of 1.25% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 6.8, was passed through, and the column was washed thoroughly with saline. The HRP/glutaraldehyde solution was rapidly filtered through this column at 4° with saline. The brown band of "activated" HRP was collected in a tube containing 5 mg of αBT with 2×10^6 cpm of ^{125}I-labeled αBT in 4 ml of saline. One molar sodium carbonate buffer, pH 9.0, was immediately added to a final concentration of 0.05 M. After 5 hr at 4°, the pH of the solution was adjusted to 7.0 with 1 M NaH_2PO_4. One molar $NaCNBH_3$ (Alpha Chemicals), a specific reagent for the reduction of Schiff bases (20), was freshly prepared in 1 M sodium phosphate buffer, pH 7.0, and was added to the reaction mixture to a final concentration of 10 mM. The solution was kept at 0° for 10–20 hr and then NH_4Cl was added to a final concentration of 20 mM. The solution was passed through a column of Sephadex G-100 with 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.4, and the fractions with both high absorbance at 403 nm (indicating HRP) and high cpm (indicating αBT) were pooled and dialyzed against 10 mM sodium acetate, pH 5.0. This mixture, containing αBT-HRP and a large amount of free HRP, was then passed through a column of CM-Sephadex C-50, the column was washed with 10 mM and 25 mM sodium acetate, pH 5.0, to elute most of the free HRP, and the αBT-HRP was eluted as a single peak with a gradient of 25–250 mM sodium acetate, pH 5.0. Polyacrylamide gel electrophoresis (21) showed that this peak was completely free of unconjugated αBT and only slightly contaminated with unconjugated HRP (Fig. 1). The yield of αBT-HRP was 10–15% of the αBT used and 25–35% of the HRP used. The molar ratio of αBT to HRP in the conjugate was 1:1, as determined by measuring radioactivity and absorbance at 403 nm. The molecular weight was estimated by gel electrophoresis to be about 48,000, as expected for a 1:1 conjugate.

Analysis of αBT-HRP Binding. Binding of αBT-HRP to Triton X-100-solubilized crude AcCh receptor from *Torpedo californica*, prepared according to Aharonov *et al.* (22), was measured by competition with ^{125}I-labeled αBT and by protection of binding sites for ^{125}I-labeled αBT. All incubations were carried out at 0° in 100 mM NaCl, 50 mM Tris·HCl at pH 7.5, 1 mM EDTA, 10 μM phenylmethylsulfonyl fluoride, 0.1% Triton X-100, with 2 mg of bovine serum albumin (BSA) per ml. To measure binding competition 10 nM ^{125}I-labeled αBT was incubated with 3 μg (protein) of the extract containing AcCh receptor, and the specified concentration of αBT-HRP in a volume of 100 μl for 10 min. The reaction was stopped by adding ammonium sulfate to 35% saturation, the precipitate was collected on glass fiber filters and washed three times with 3 ml of 35% saturated ammonium sulfate solution, and the ra-

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Abbreviations: AcCh, acetylcholine; αBT, α-bungarotoxin; HRP, horseradish peroxidase; IPL, inner plexiform layer; BSA, bovine serum albumin; DAB, 3,3′-diaminobenzidine.

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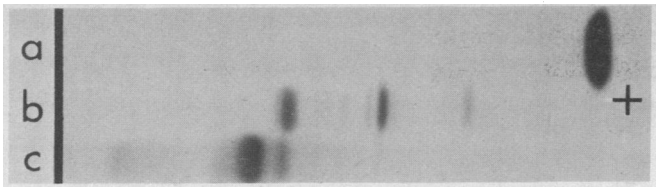

FIG. 1. Polyacrylamide gel electrophoresis of αBT-HRP and the proteins used in its synthesis. Electrophoresis was performed on a slab gel gradient of 10–20% polyacrylamide in 0.1% sodium dodecyl sulfate as described by Maizel (21) for acidic proteins. The gels were stained with Coomassie Blue. Channel a contains 5 μg of the αBT used. Channel b contains 5 μg of the HRP used. Contaminants of lower molecular weight are seen. Channel c contains 6 μg of the αBT-HRP obtained after CM-Sephadex chromatography. It is contaminated with free HRP. Heavy line indicates origin.

dioactivity was measured by scintillation counting as described by Aharonov *et al.* (22). Measurement of binding site protection was carried out in the same manner, except that the extract containing AcCh receptor was preincubated with αBT-HRP for 20 min, then 10 nM ^{125}I-labeled αBT was added, and incubation was continued for another 10 min.

The binding of αBT-HRP to the neuromuscular junction of mouse diaphragm was assayed histochemically. Cryostat sections, 16–20 μm thick, of mouse diaphragm were mounted on slides and incubated 90 min at 20° with various concentrations of αBT-HRP, with or without the addition of 1 mM decamethonium or 1 μM unlabeled αBT, all in Eagle's basal medium (Gibco) with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) instead of bicarbonate and BSA at 2 mg/ml (10). Some sections were preincubated with 10 nM unlabeled αBT in the same medium at 37° for 1 hr, then washed briefly with medium before incubation with 3 μM or 0.3 μM αBT-HRP. The sections were then washed in several changes of the same medium at 4° for 1 hr, washed briefly with medium without BSA, fixed for 1 hr at 4° with 2% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.4, washed with several changes of the same buffer and then with 0.05 M Tris·HCl at pH 7.6, and incubated 1 hr at 20° in the dark in a solution of 3,3'-diaminobenzidine (DAB) at 0.1–0.2 mg/ml with 0.001–0.003% H_2O_2, respectively, in the same Tris buffer (23).

Histochemical Procedure. Retinas of White Leghorn hens were treated by injecting the specified compounds into the vitreous body of the eye. Eyes were injected with αBT-HRP at either 9 μM or 3 μM dissolved in 150 μl of isotonic salt solution (24) with BSA at 2 mg/ml. The volume of the vitreous body was about 1.5 ml. Because the injection site was close to the retina, different regions of the retina were initially exposed to αBT-HRP at an approximate range of concentrations between the injected concentration and 10-fold lower. Injection of 9 μM αBT-HRP did not seem to produce darker or more frequent synaptic labeling than 3 μM. Control injections, into the eyes of different hens, contained one of the following: 9 μM αBT-HRP with 1 μM unlabeled αBT; 3 μM αBT-HRP with 1 μM unlabeled αBT, 3 μM αBT-HRP with 1 mM *d*-tubocurarine, 10 μM HRP, or isotonic saline with BSA at 2 mg/ml. After 3 hr the hens were anesthetized with ether, the eyes were removed, and the animals were dispatched. The front of the eye was removed along with the vitreous body, and the remaining portion was washed in a solution of 90% Eagle's basal medium and 10% fetal calf serum equilibrated with 95% O_2/5% CO_2, followed by washes in the same solution without serum at 4° for a total of 15 min. The tissue was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, with 2 mM $CaCl_2$ at 4° for 1 hr, and the retina was removed and washed in several changes of 0.1 M sodium cacodylate, pH 7.3, and in 0.05 M Tris·HCl, pH 7.6. After 15 min preincubation at 20° with DAB at 0.5 mg/ml in 0.05 M Tris·HCl, pH 7.6, the retina was incubated for an additional 45 min in the same solution with the addition of 0.01% H_2O_2 (24), then washed with the same Tris buffer and with 0.1 M sodium cacodylate buffer, pH 7.3. The tissue was postfixed for 1 hr at 4° with 1% OsO_4 in the same buffer, dehydrated in ethanol, and embedded in Epon 812.

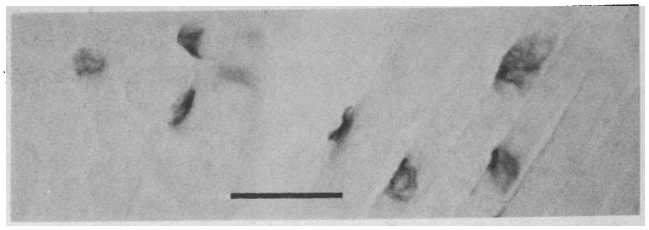

FIG. 2. Mouse diaphragm endplates stained with 0.3 μM αBT-HRP. Bright field photomicrograph. Bar represents 50 μm.

Sampling and Staining Procedures for Electron Microscopy. The initial observations were made on thin sections that had *not* been stained with uranyl acetate or lead citrate, allowing maximum contrast between electron density due to DAB reaction product and that of the tissue. Because staining the section was required for optimum delineation of synaptic structure, but obscured the identification of DAB reaction product, the following methods were developed: (*i*) Sections were stained for 5 min with saturated aqueous (about 5%) uranyl acetate. This clearly stained the bipolar synaptic ribbons and submembrane material with relatively little effect on membranes and vesicles. (*ii*) Serial sections were mounted on Formvar/carbon films on slot grids. One section on each grid was carefully scanned with the electron beam. When all the sections were subsequently treated with uranyl acetate and lead citrate the exposed section did not take up stain but the adjacent sections did. Electron micrographs were obtained of the same synapses in the exposed (unstained) section and the stained adjacent serial sections. (*iii*) Sections were stained with uranyl acetate and lead citrate after brief exposure to the electron beam. This gave less tissue staining than in unexposed sections.

RESULTS

Binding of αBT-HRP to Nicotinic AcCh Receptors. In binding competition, 100 nM and 600 nM αBT-HRP blocked 21% and 34%, respectively, of the binding of 10 nM ^{125}I-labeled αBT to solubilized nicotinic AcCh receptors from *Torpedo* electric organ. Using preincubation with αBT-HRP, 34% and 85% of 10 nM ^{125}I-labeled αBT binding was blocked by 100 nM and 600 nM αBT-HRP, respectively. The results of binding competition were comparable to those obtained with chick embryo muscle homogenate as a source of receptor (W. Catterall, unpublished results). These results indicated that the affinity of αBT-HRP for nicotinic AcCh receptor was substantially lower than that of ^{125}I-labeled αBT, but that αBT-HRP was capable of competing for the receptor sites and could protect a high proportion of available sites against ^{125}I-labeled αBT binding.

With the histochemical assay, endplates of mouse diaphragm were distinctly stained by 50 nM to 1 μM αBT-HRP (Fig. 2). Background staining was low, and the intensity of endplate staining increased with increasing concentration of αBT-HRP. No endplate staining was obtained after preincubation with 10 nM αBT or when either 1 mM decamethonium or 1 μM αBT was added with the αBT-HRP. Therefore, αBT-HRP bound

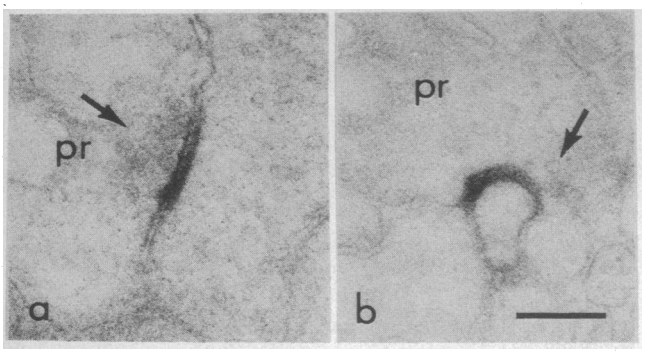

FIG. 3. Unstained sections of amacrine synapses labeled with αBT-HRP. DAB reaction product is seen on the postsynaptic membrane, in the synaptic cleft, and on the presynaptic membrane. The presynaptic processes (pr) contain faintly visible clusters of synaptic vesicles (arrows). (*a*) Synapse with a "flat" synaptic cleft. (*b*) Synapse with a "round" synaptic cleft and a relatively small postsynaptic process. Bar represents 0.25 μm.

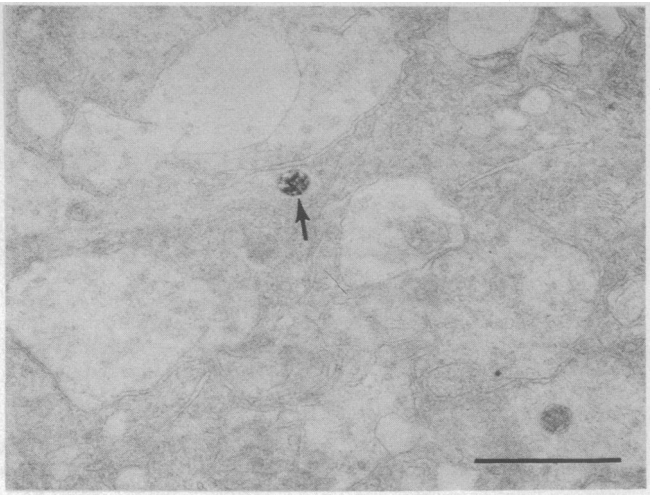

FIG. 4. Unstained section of an IPL region in a control eye injected with 3 μM αBT-HRP and 1 mM *d*-tubocurarine. A vesicular inclusion (arrow) containing DAB reaction product is seen within the postsynaptic process of an unlabeled synapse. Bar represents 1.0 μm.

to diaphragm with the specificity expected for binding to nicotinic AcCh receptors.

Electron Microscopy. Synapses were defined as regions of neuronal contact with increased submembrane density, a regular, usually widened intercellular cleft, and an accumulation of vesicles close to one side of the cleft. DAB reaction product was found on the postsynaptic membrane, in the synaptic cleft, and on the presynaptic membrane of synapses in the inner plexiform layer (IPL) of the αBT-HRP-treated retinas (Fig. 3). More reaction product was usually found on the postsynaptic than the presynaptic side of the cleft (Figs. 3*a*, 6, and 8). Occasionally, reaction product was observed on nonsynaptic regions of nerve and glial membranes or in the intercellular space; but here the reaction product appeared as small discrete spots (about 200 Å in diameter). Reaction product was also seen occasionally on the membranes of damaged cell processes, and frequently in membrane-bound inclusions within nerve and glial processes throughout the retina (Fig. 4). Little or no reaction product was found in retinas from eyes injected with isotonic salt solution containing BSA. The presence of nonsynaptic DAB reaction product did not depend on the specific binding activity of the αBT moiety of the αBT-HRP; control retinas from eyes injected with a mixture of 3 μM or 9 μM αBT-HRP and 1 μM αBT, or 3 μM αBT-HRP and 1 mM *d*-tubocurarine (Fig. 4), conditions which should block binding of the αBT-HRP to nicotinic AcCh receptors, as well as retinas from eyes injected with 10 μM HRP alone, had DAB reaction product at the nonsynaptic sites just described. However, labeled synapses were seldom seen in these control retinas and these few synapses had scant deposits of reaction product. We conclude that synaptic binding of αBT-HRP is specific for nicotinic AcCh receptors; and that the receptors of retinal neurons appear to be concentrated in the synapse.

Most synapses in the IPL were classified into two main types, described in detail elsewhere (13, 25, 26): bipolar cell (bipolar) synapses, identifiable by the presence of synaptic ribbons (Fig. 5), and "conventional" synapses similar to other synapses found throughout the central nervous system (Figs. 3 and 6–8), whose presynaptic processes have no ribbons. The latter have been identified as amacrine cell (amacrine) synapses (13, 25, 26).

Bipolar synapses (Fig. 5) as well as amacrine synapses (Figs. 3 and 6–8) throughout the IPL bound αBT-HRP. Labeled amacrine synapses were classified into two types, those with a "flat" synaptic cleft with relatively large pre- and postsynaptic processes (Figs. 3*a* and 6–8), and those with a "round" synaptic cleft in which a small postsynaptic process fit into an indentation in the larger presynaptic process (Fig. 3*b*). Some of the postsynaptic processes of bipolar synapses that bound αBT-HRP were large and contained scattered clear vesicles, while others were smaller and lacked vesicles. One of the two postsynaptic processes usually found at these synapses was heavily labeled and the other was unlabeled or lightly labeled (Fig. 5).

The neuronal processes of αBT-HRP-labeled synapses in the IPL were involved in several types of synaptic relationships. (*i*) Some labeled amacrine synapses were in serial relation to unlabeled amacrine synapses (Fig. 7). (*ii*) Amacrine processes,

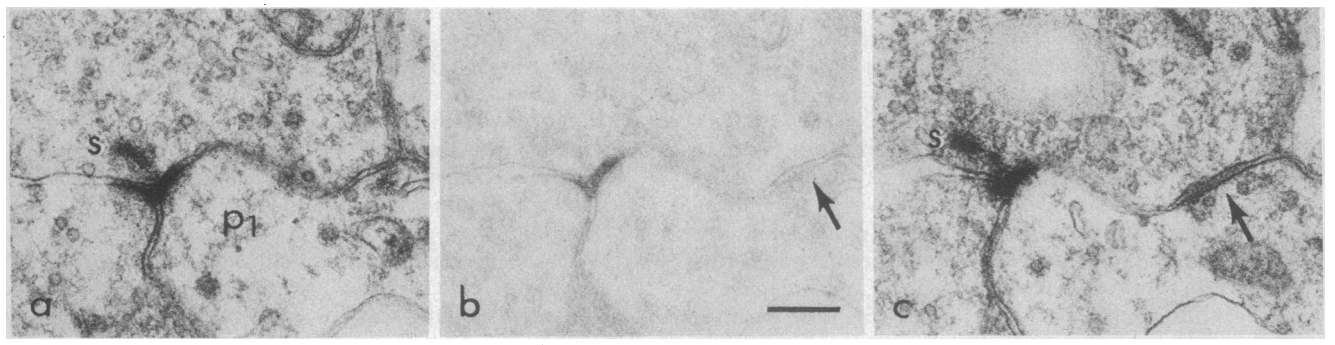

FIG. 5. Serial sections of a bipolar synapse labeled with αBT-HRP. The section in *b* was first exposed to the electron beam, then stained with uranyl acetate and lead citrate and examined. The sections shown in *a* and *c* were first stained and then examined. The presynaptic process contains the characteristic synaptic ribbon (s). One postsynaptic process (p_1) is labeled more heavily than the other. Both postsynaptic processes contain clear vesicles, and p_1 forms an unlabeled reciprocal (arrows) synapse with the bipolar cell process. Bar represents 0.25 μm.

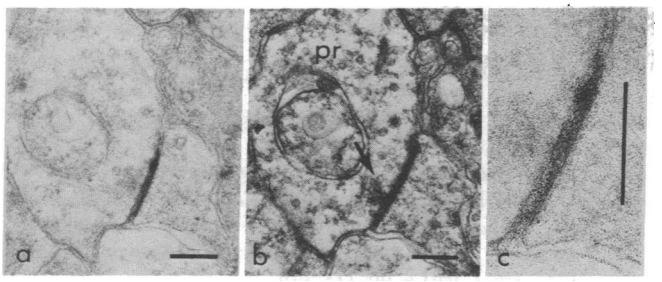

FIG. 6. Serial sections of an amacrine synapse labeled with αBT-HRP. The sections were briefly stained with uranyl acetate. The section shown in *a* and *c* was first exposed, then stained with lead citrate. The section shown in *b* was first stained with lead citrate, then examined. The presynaptic process (pr) contains a mitochondrion, scattered clear vesicles, and a cluster of vesicles (arrow) at the synapse. Panel *c* shows the synaptic cleft region at higher magnification. The heaviest accumulation of DAB reaction product appears on the postsynaptic side of the synaptic cleft. Bars represent 0.25 μm.

which were labeled at a bipolar synapse, made unlabeled reciprocal synapses with the same bipolar processes (Fig. 5). (*iii*) A single amacrine process could be the presynaptic element of labeled synapses and adjacent unlabeled synapses (Fig. 8). (*iv*) Conversely, a single process could be the postsynaptic element of both labeled and unlabeled synapses.

The proportion of retinal IPL synapses with nicotinic AcCh receptor was estimated by counting the αBT-HRP-labeled synapses in electron micrographs of unstained sections and in sections stained briefly with uranyl acetate to stain synaptic ribbons. Random fields as well as a complete scan between the inner nuclear layer and the ganglion cell layer of a portion of the IPL were taken. The area sampled was 1940 μm^2, containing approximately 830 synapses. The total number of bipolar and amacrine synapses per unit area of the sections was counted in random fields of sections stained with uranyl acetate and lead citrate. A unit containing a ribbon and its associated postsynaptic processes was counted as a single bipolar synapse. Five to seven percent of all synapses were labeled with αBT-HRP. Four to six percent of the amacrine synapses and 14–20% of the bipolar synapses were labeled, but because the bipolar synapses constituted only 10% of all synapses, about 75% of the labeled synapses were of the amacrine type.

DISCUSSION

Results of previous studies (9, 10) suggested that nicotinic AcCh receptors are associated with synapses in the developing and mature chick retina. In brief, the present results indicate that nicotinic AcCh receptors in retina are concentrated in synapses. Specific synapses in the adult chick retina will bind αBT-HRP. The observed binding properties of αBT-HRP indicate that the synaptic binding sites are nicotinic AcCh receptors. Lentz *et al.* (27, 28) have similarly used an αBT-HRP conjugate to label the nicotinic AcCh receptors of the neuromuscular junction. If extrasynaptic nicotinic AcCh receptors are present in the adult chick retina, they are apparently at a much lower density than synaptic receptors.

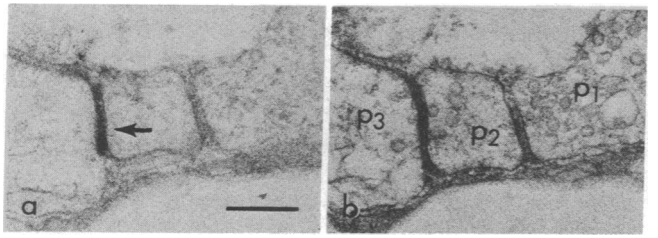

FIG. 7. Serial sections of two amacrine synapses in series, one synapse labeled with αBT-HRP (arrow) and the other unlabeled. Sequence of exposure, staining, and examination is as in Fig. 5 *a* and *b*. Process p_1 appears to be presynaptic to p_2, which in turn is presynaptic to p_3. Bar represents 0.25 μm.

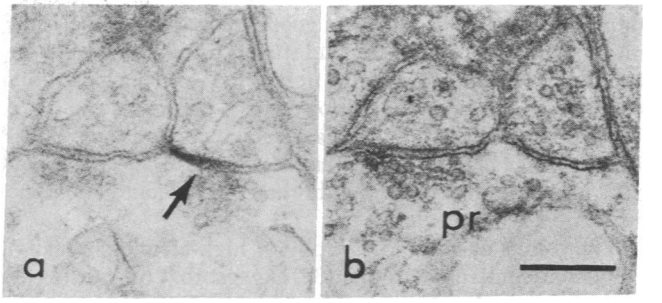

FIG. 8. Serial sections of an amacrine process (pr) that formed labeled (arrow) and unlabeled synapses with different postsynaptic processes. Sequence of exposure, staining, and examination is as in Fig. 5 *a* and *b*. Both postsynaptic processes contain clear vesicles. Bar represents 0.25 μm.

The heaviest accumulation of DAB reaction product in the retinal synapses was on the postsynaptic side of the synaptic cleft, suggesting postsynaptic location of the nicotinic AcCh receptors, but reaction product also appeared on the presynaptic membrane in parts of many synapses, especially the heavily labeled ones. This could result from diffusion of reaction product across the synaptic cleft (5, 29). However, because the relative affinity of the DAB reaction product for the pre- and postsynaptic membranes is unknown, it was not possible as a rule to distinguish between pre- and postsynaptic nicotinic AcCh receptor sites.

Results of studies on αBT binding (9, 10), acetylcholine release (16), and choline acetyltransferase activity (15) have suggested that amacrine or bipolar cells in the vertebrate retina make cholinergic synapses. The present results indicate that both of these cell types make cholinergic synapses. The results also allow preliminary classification of the postsynaptic cell types in cholinergic synapses. Labeled processes that are postsynaptic at a bipolar ribbon synapse and also form a reciprocal synapse with the bipolar cell are almost certainly amacrine cell processes (13, 25, 26). Neurophysiological studies (30) have provided evidence for the presence of nicotinic AcCh receptors on ganglion cells in the rabbit retina. Small, round postsynaptic processes without vesicles, postsynaptic to bipolar and amacrine cells, resembled ganglion cell dendrites. These processes were often labeled, suggesting that some ganglion cell dendrites in chicken retina have nicotinic AcCh receptors.

Labeled synapses were found in a variety of relationships with unlabeled synapses. Only one of the two postsynaptic processes in most bipolar ribbon synapses was clearly labeled. If one assumes that a bipolar neuron that forms a *nicotinic* cholinergic synapse secretes only AcCh, then it is likely that the unlabeled or slightly labeled process has a predominance of *muscarinic* AcCh receptors. This is a reasonable interpretation in view of the high concentration of muscarinic AcCh receptors known to exist in the chicken retina (H. Sugiyama, M. P. Daniels, and M. Nirenberg, unpublished results). The same interpretation may apply when a single amacrine process is the presynaptic element of both labeled and unlabeled synapses, as shown in Fig. 8. Failure of αBT-HRP to penetrate the synaptic cleft could also explain this result. However, labeled synapses showed no sign of structural disruption that might allow preferential binding as compared to unlabeled neighboring synapses; the presence of labeled vesicular inclusions

throughout the retina indicated good penetration of αBT-HRP into the intercellular spaces; and studies using higher concentrations of HRP alone in retina *in vivo* (31) and in chick retina cell aggregates *in vitro* (Z. Vogel, G. J. Maloney, and M. P. Daniels, unpublished results) have shown penetration by HRP in most, if not all, synapses within 15–90 min. αBT-HRP, about 20% higher in molecular weight than HRP, should also penetrate most synaptic clefts.

The results of autoradiography of high-affinity uptake of [^{3}H]choline into chick retina (32) suggest that 6% of the cells in the inner nuclear layer, half located as expected for amacrine cells, half as for bipolar cells, are cholinergic. In the present study, 4–5% of the amacrine synapses and 14–20% of the bipolar synapses were labeled. Therefore, these cholinergic amacrine and bipolar cells appear to form a significant proportion of the synapses in the IPL of chick retina.

Note Added in Proof. After this article went to press, we learned of a study on the binding of an α-bungarotoxin-horseradish peroxidase conjugate to synapses in rat brain (Lentz, T. L. & Chester, J., *J. Cell Biol.*, in press).

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