

LOCALIZATION OF ACETYLCHOLINE RECEPTORS IN CENTRAL SYNAPSES

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ABSTRACT

The localization of cholinergic receptors in brain synaptosomes and in synapses of the midbrain reticular formation and hypothalamic preoptic nucleus has been demonstrated by means of a horseradish peroxidase- α -bungarotoxin (HRP- α -Btx) conjugate. Only a small proportion of the total number of synapses was reactive. Axon terminals of reactive synapses contained primarily small clear vesicles, while synapses characterized by large numbers of dense core vesicles were unreactive. Toxin-binding sites were found to occur in a thickened zone of the postsynaptic surface. This procedure can be employed to study the regional distribution and localization of nicotinic receptor sites in the central nervous system.

KEY WORDS acetylcholine receptors
synapse · synaptic membrane · central
nervous system · cytochemistry

The snake neurotoxin α -bungarotoxin (α -Btx) has proven useful in the localization of acetylcholine receptors (AChR) by virtue of its ability to bind specifically and virtually irreversibly to nicotinic receptor sites (15, 18). By using radioactively labeled α -Btx or markers attached to the toxin, it has been possible to determine the location and number of these cholinergic receptor sites. For example, the toxin has been used to detect receptors in skeletal muscle (4, 6, 25), sympathetic ganglia (8), and retina (35, 36).

The brain contains nicotinic, muscarinic, and mixed cholinergic synapses, with the majority being of the muscarinic type (3, 34). Several studies have demonstrated binding of α -Btx to brain tissue or fractions indicating that α -Btx can be used for the localization of nicotinic receptors in the central nervous system (1, 5, 12, 17, 19, 20, 24, 28, 29, 31, 33). Toxin binding is highest in fractions containing synaptosomes (5, 12, 29).

We have described recently a procedure in which AChR are localized with α -Btx conjugated directly with horseradish peroxidase (HRP) (16). This procedure permits the localization of sites of toxin binding at high resolution with the electron microscope. This technique has been applied to the neuromuscular junction (16), and the present paper reports on its suitability for the localization of AChR in synapses of the central nervous system.

MATERIALS AND METHODS

Preparation of HRP- α -Btx Conjugate

Bungarus multicinctus venom was obtained from the Sigma Chemical Co. (St. Louis, Mo.) or the Miami Serpentarium (Miami, Fla.). α -Btx was separated from the venom on a carboxymethylcellulose column according to the method of Clark et al. (2). Conjugation of α -Btx to HRP was achieved utilizing the method of Nakane and Kawaoi (21). HRP (40 mg) and α -Btx (8 mg) were used in equimolar ratios in order to favor 1:1 conjugates. The products of conjugation were separated by column chromatography on Ultragel AcA44 (LKB Instruments, Inc., Hicksville, N. Y.) and fractions from an apparent

mol wt of 42,000 to 85,000 daltons were pooled and utilized for the cytochemical localization of AChR. This fraction was tested physiologically and produced a post-synaptic blockade at frog sartorius neuromuscular junction similar to that obtained with native α -Btx, except that longer exposure to the conjugate was required. Miniature end plate potentials and muscle twitch were blocked after 2-h exposure to the conjugate, and end plate potentials were abolished after 3–4 h. Native α -Btx (10^{-6} M) blocked the muscle twitch in 15 min, and end plate potentials after 20 min. These studies indicate that the conjugate retains activity although its potency is decreased, possibly because the coupling procedure utilizes some of the lysine residues of α -Btx essential for its biologic activity (see reference 16). Vogel et al. (35) have analyzed the binding of HRP- α -Btx conjugate to solubilized nicotinic AChR from *Torpedo* electric organ. They found that the affinity of the conjugate for the receptor is lower than that of 125 I-labeled α -Btx, but that it is capable of competing for receptor sites and protects a high proportion of sites against 125 I-labeled α -Btx binding. Peroxidative activity of the conjugate was determined by measuring the rate of decomposition of hydrogen peroxide with *o*-dianisidine as hydrogen donor (38). Conjugation was found not to produce a decrease in peroxidative enzymatic activity. Further details of the conjugation procedure have been reported (16).

Synaptosome Preparation and Incubation

Whole rat brains were homogenized with a glass homogenizer in cold 0.32 M sucrose (10% wt/vol). A P₂ fraction (containing myelin fragments, nerve ending particles, and mitochondria) was prepared according to the method of Gray and Whittaker (7). The pellet was resuspended in 3 ml of 0.32 M sucrose using a Vortex mixer (Scientific Industries, Inc., Springfield, Mass.). 12–15 vol of Ringer's solution were added slowly to the suspension with mixing. The fraction was centrifuged at 9,000 g for 5 min at 4°C and washed twice with Ringer's solution in this manner.

For localization of AChR, the pellet was resuspended in 2 ml of Ringer's solution minus phosphate and containing 0.2 ml of the conjugate (produces a final concn of 10 mM phosphate). Controls consisted of preincubation for 1 h in 5 ml of Ringer's solution containing 0.4 mg of native α -Btx followed by centrifugation and resuspension in the conjugate or by suspension in Ringer's solution alone. The preparations were incubated for 1½ h at 4°C with occasional gentle mixing. After incubation, the preparations were washed three times by suspension in Ringer's solution and centrifugation at 9,000 g for 5 min. The pellets were fixed for 60–90 min in 3% glutaraldehyde. Small pieces were excised from the pellets and processed as described below for tissue blocks.

Tissue Incubation and Cytochemistry

Young rats were anesthetized with ether and the brains removed immediately and placed in Ringer's solu-

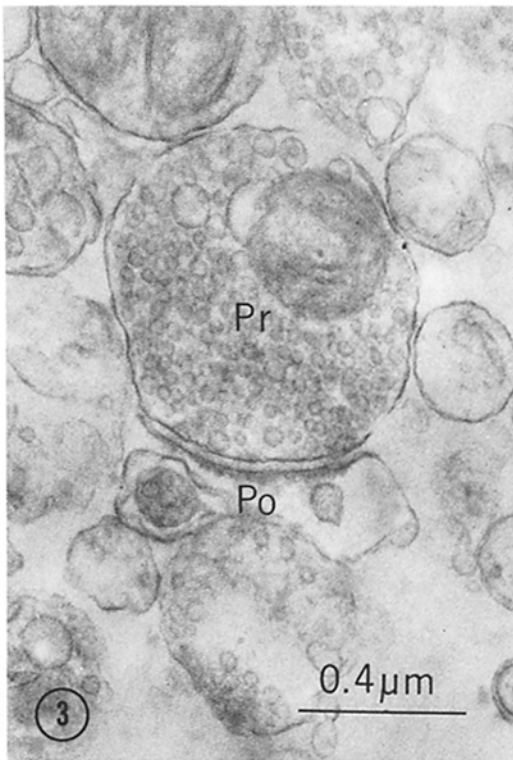
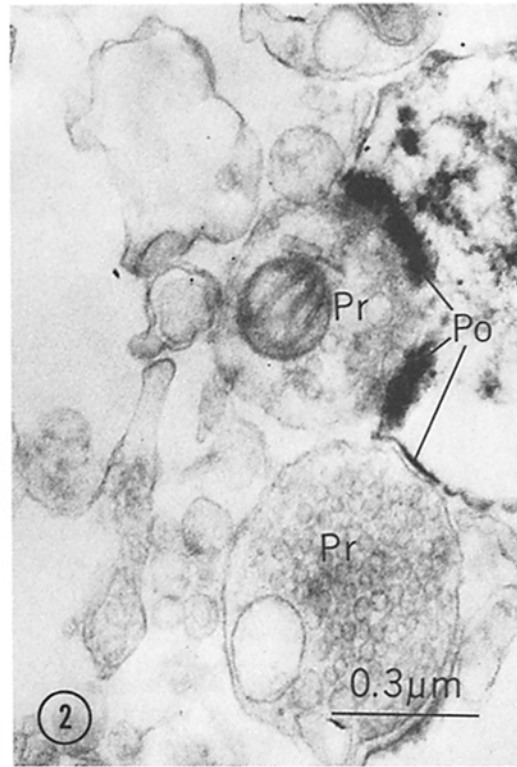
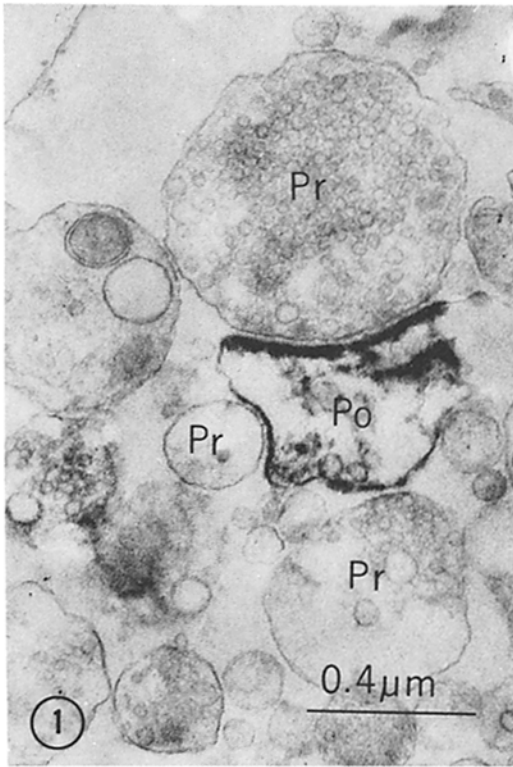
tion. Frontal slices (~2 mm thick) were made with a razor blade. Slices were employed to obtain maximum exposure and penetration of the conjugate into the tissues, since α -Btx does not readily cross the blood-brain barrier (15). The unfixed slices were incubated for 2 h in the conjugate (diluted 10-fold in Ringer's solution minus phosphate) with gentle agitation at room temperature and gassed with 95% O₂, 5% CO₂. Some slices were preincubated in native α -Btx (1 mg/6 ml Ringer's) for 2 h and incubated in the conjugate or were incubated in Ringer's solution alone. After incubation, the slices were rinsed in Ringer's solution (with phosphate) for 2 h with changes every 15 min. Tissues were then fixed in 3% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 1 h and rinsed in buffer with changes for ~30 min.

The brain slices and synaptosome pellet blocks were reacted for peroxidase activity by incubation in 3,3'-diaminobenzidine (DAB) (Sigma Chemical Co.) (50 mg/100 ml Tris buffer, pH 7.2) and H₂O₂ (0.01%) for 60–90 min. Tissues and pellet blocks were rinsed in buffer and small blocks were excised from the brain slices. Several regions of brain were surveyed and the midbrain reticular formation and hypothalamic preoptic nucleus were selected for study of synapses. Blocks were taken from the edges of the tissue slices. In the case of the preoptic nucleus, blocks were taken from the region adjacent to the third ventricle and included the periventricular zone of this nucleus. The blocks were dehydrated, infiltrated, and embedded in Epon 812. Thin sections were viewed unstained with a Hitachi 8B or HU11-ES electron microscope.

RESULTS

Reactive synapses were observed in both synaptosome preparations (Figs. 1 and 2) and tissue blocks (Figs. 5–9). The reaction product for peroxidase activity appeared as a dense, fine-textured deposit, and occurred primarily on the postsynaptic portion of the synapses. Other membranes, myelin, synaptic vesicles, and most mitochondria were unreactive. Sites of reaction product occurring in both HRP- α -Btx-treated tissues and control preparations, and interpreted to represent endogenous peroxidatic activity, are described below.

Reactive synapses were similar in structure and consisted of a presynaptic bouton and postsynaptic element (Figs. 1, 2, 5–9). The axon terminals contained numerous synaptic vesicles, 500 Å in diam, with clear contents. Mitochondria and a few larger dense-core vesicles or granules usually were present as well. The synaptic cleft was about 150 Å in width and contained moderately dense material. Finely filamentous, moderately dense material was applied to the inner aspects of the postsynaptic, and to a lesser extent, the presynaptic mem-



branes. The postsynaptic element sometimes contained a mitochondrion but generally contained few organized structures. In contrast, synapses in which the axon terminal contained a large number of larger dense-core vesicles were unreactive (Fig. 7). These terminals often contained some small, clear vesicles as well.

In reactive synapses, activity was primarily localized to the postsynaptic membrane. The reactive region was located opposite the presynaptic membrane and extended laterally a short distance beyond the region of synaptic contact (Fig. 6). Reaction product occurred in a uniform band which included the postsynaptic membrane (Figs. 6, 8, and 9). The width of the reactive band (125 Å) exceeded the width of the plasma membrane in control preparations (75 Å) by about 50 Å.

In intensely reacted specimens, reaction product extended into the postsynaptic density (Figs. 7 and 9). This appearance could be due to build-up of reaction product with trapping, and adherence by the filamentous network forming the postsynaptic density. In addition, in a few instances, some portions of the presynaptic axonal membrane were reactive. Reaction product was localized to the plasma membrane at the region of synaptic contact (Fig. 9). Some staining also occurred in the membrane forming the sides of the terminal. Although the localization of reaction product in the axolemma was similar to that observed in the presynaptic axon terminals of the neuromuscular junction (16), it was seen in only a few synapses in heavily reacted preparations. Thus, no conclusions can be made at this time as to whether this staining is indicative of presynaptic receptors in central synapses.

Controls in which synaptosomes (Figs. 3 and 4) and tissues (Figs. 10 and 11) were incubated in

Ringer's solution alone or were pretreated with native α -Btx to block specific cholinergic-binding sites before incubation in the conjugate, did not show reaction product at the region of synaptic contact. It should be noted though, that the finely filamentous material applied to the inner aspects of pre- and postsynaptic membranes varies in density and in some cases is moderately dense in control preparations (Figs. 10 and 11). In addition, in controls, the pre- and postsynaptic membranes in the region of synaptic contact are somewhat darker than nonsynaptic membranes (Figs. 10 and 11). These inherent densities are to be distinguished from the densities due to addition of reaction product. The latter is extremely dense to opaque. In addition, in controls, the postsynaptic membrane could be seen distinctly and was 75 Å in width (Figs. 10 and 11), while in reactive specimens, postsynaptic reaction product occurred in a band 125 Å in width.

While staining was not seen in the postsynaptic membrane of control preparations, activity occurred in other regions in both control and experimental preparations. Activity was most prominent in membrane-bounded granules and vacuoles within neurons and their processes, glia, and ependymal cells. These structures may represent lysosomes or peroxisomes. Some mitochondria were reactive and erythrocytes were increased in density. Some processes contained dense particulate material of unknown nature, particularly in the α -Btx controls. Sites of endogenous peroxidatic activity in the brain have been described (11, 37). Heme enzymes such as peroxidase, catalase, and cytochromes are capable of catalyzing the oxidation of DAB. Another source of activity is conjugate trapped within cellular processes and not washed out during rinsing.

FIGURE 1 Synaptosomes after incubation in HRP- α -Btx conjugate for 2 h and reaction for HRP. Reaction product is localized to the postsynaptic element (*Po*) upon which presynaptic profiles (*Pr*) terminate. $\times 56,000$.

FIGURE 2 Synaptosomes after incubation in the conjugate and reaction for HRP. Activity occurs postsynaptically (*Po*) at regions of synaptic contact. *Pr*, presynaptic element. $\times 65,000$.

FIGURE 3 Synaptosome control preincubated in native α -Btx for 1 h, incubated in the conjugate for 2 h, and reacted for HRP. No activity occurs at the region of synaptic contact. *Pr*, presynaptic profile; *Po*, postsynaptic profile. $\times 55,000$.

FIGURE 4 Synaptosome control showing absence of reaction product at regions of synaptic contact (*Sy*). Moderately dense material is applied to the inner aspects of the pre- and postsynaptic membranes. $\times 54,000$.

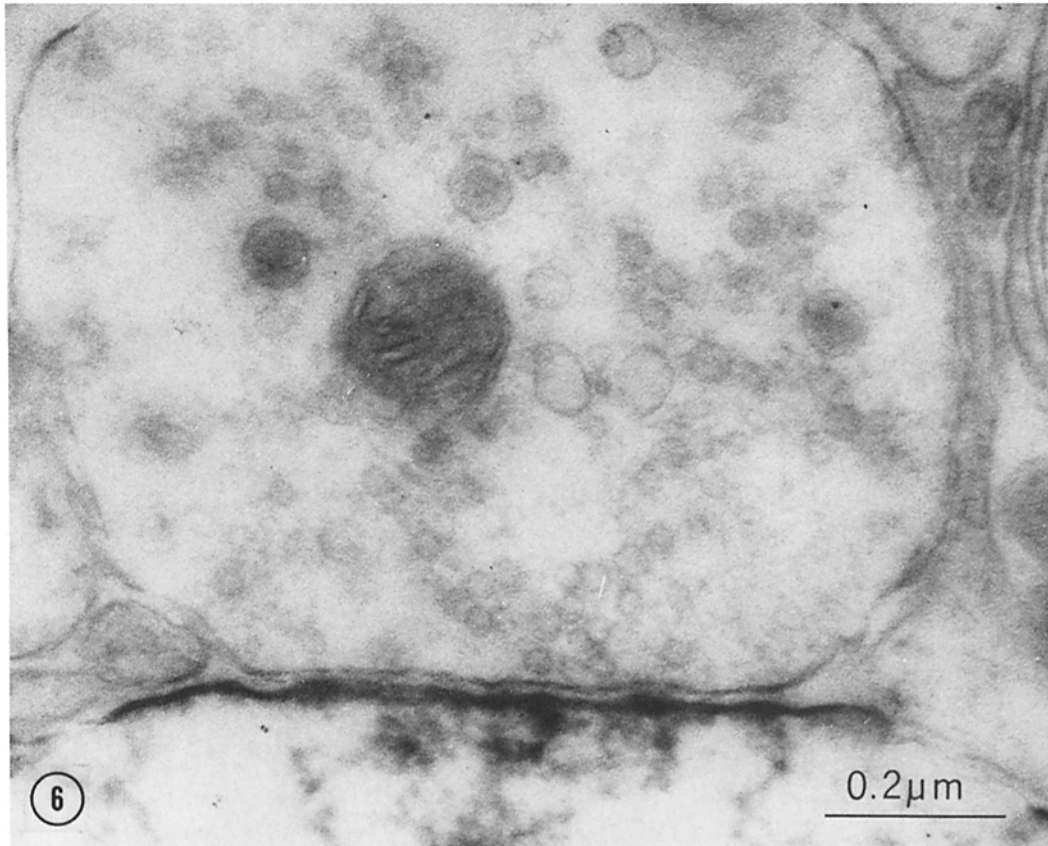
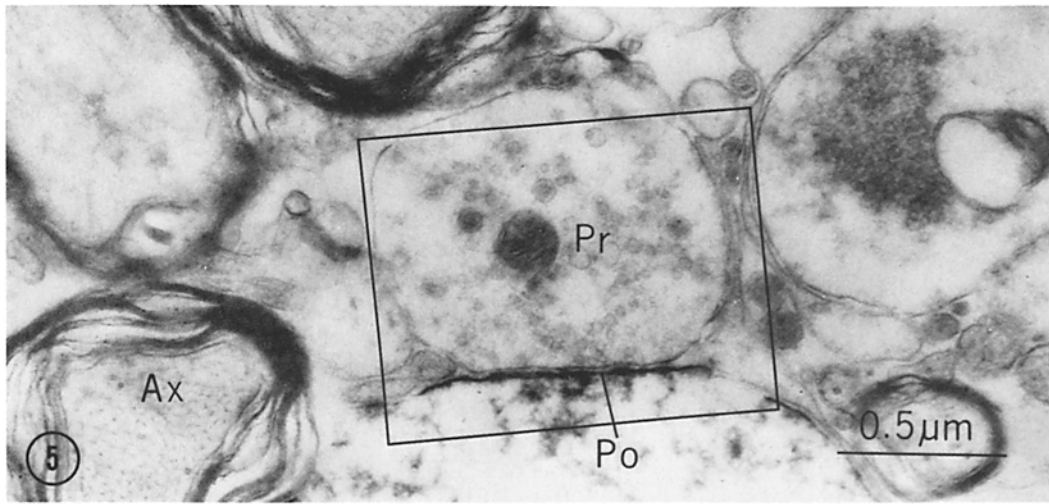


FIGURE 5 Midbrain reticular formation after procedure for localization of AChR. Activity occurs at a synapse and is localized to the postsynaptic membrane (*Po*). Other regions including the presynaptic terminal (*Pr*) and axons (*Ax*) are unreactive. $\times 45,000$.

FIGURE 6 Higher magnification of area enclosed in box in Fig. 5. Activity is restricted to the postsynaptic membrane opposite and extending a short distance beyond the region of synaptic contact. Note that the reactive region is thicker than unreactive membrane. $\times 120,000$.

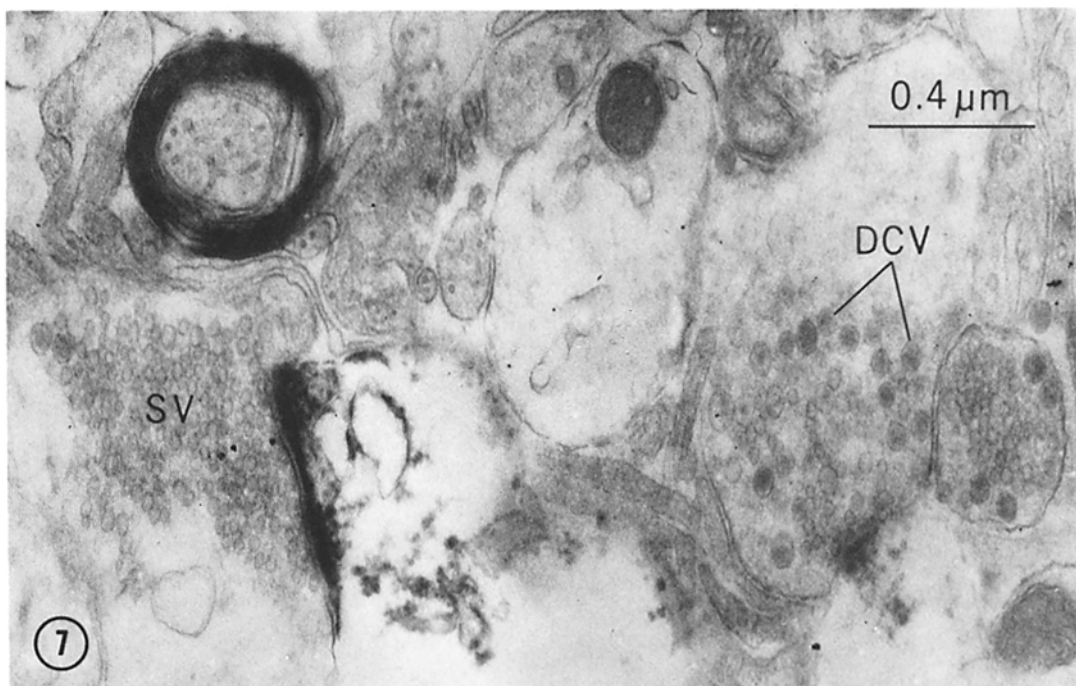


FIGURE 7 Preoptic nucleus of hypothalamus (neuropil of periventricular zone) reacted for AChR. The postsynaptic region of the synapse on the left is intensely reactive. The axon terminal of this synapse contains predominantly small clear synaptic vesicles (SV). This synapse is considered to be cholinergic. The synapse on the right is unreactive and contains larger dense-core vesicles (DCV) along with smaller clear vesicles. $\times 54,500$.

As in the case of muscle, staining of intact brain tissue with this technique is variable, with some samples containing reactive synapses and others not. Furthermore, when present, reactive synapses were located near the edges of the blocks where tissue preservation was not always optimal and cell relationships were disrupted. Such variability may be due to the relatively large size of the conjugate (mol wt 48,000 or larger) which could result in a low rate of diffusion and penetration into the tissues, and to the length of time necessary for incubation and washing of the unfixed tissues.

DISCUSSION

Nicotinic acetylcholine receptor sites have been identified in synapses of the reticular formation of the midbrain and preoptic nucleus of the hypothalamus. Also employing an HRP- α -Btx conjugate, Jensen et al. (10) have reported binding to the subsynaptic membrane in the optic tectum. Vogel et al. (35) found that 5-7% of the synapses in the inner plexiform layer of the chicken retina bound the conjugate. They observed staining on the post-

synaptic side of the synapses and in the presynaptic membrane as well.

In the present study, reaction product was localized primarily to a thickened region on the postsynaptic surface. A similar thickened postsynaptic zone of reaction product has been observed on the upper portions of junctional folds of the neuromuscular junction (16). Such a localization may be consistent with a transmembrane location of receptors projecting a short distance into the synaptic cleft, although caution is required in interpreting the exact site of localization of reaction product relative to the membrane. The oxidized DAB may not lie precisely at the site of the receptor, due to the size of the conjugate molecule bound to the receptor and to accumulation, trapping, or adsorption of reaction product. However, at the neuromuscular junction, the reactive zone coincides with the region where specializations suggested to represent the morphological counterpart of the AChR have been described (9, 23, 26, 27). Similarly, aggregations of particles have been described in the outer leaflet of the postsynaptic membranes of some central synapses (13, 14, 22,

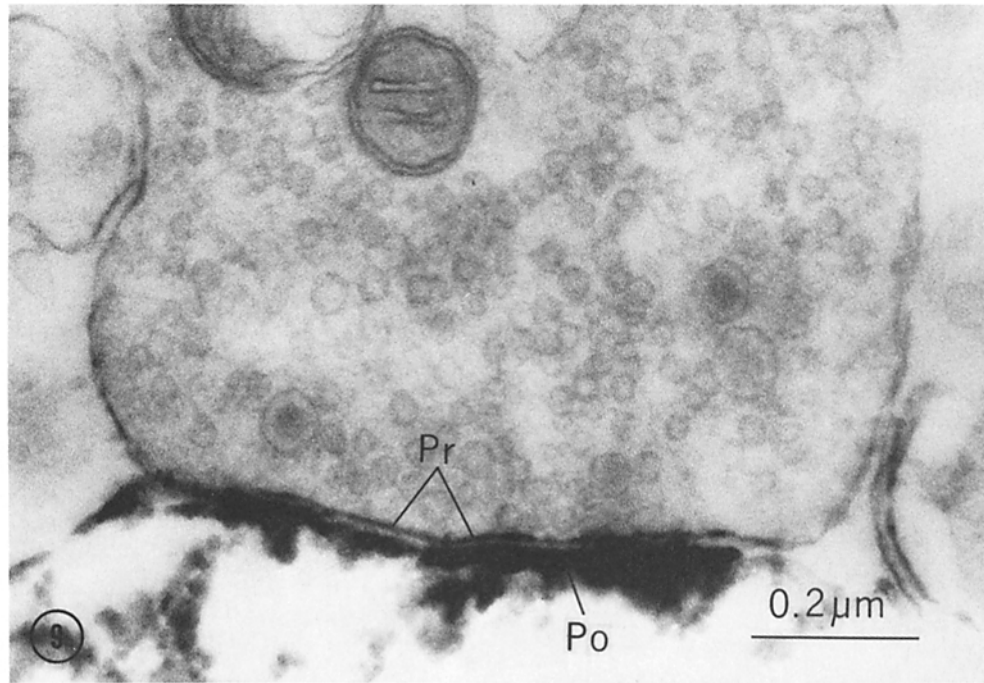
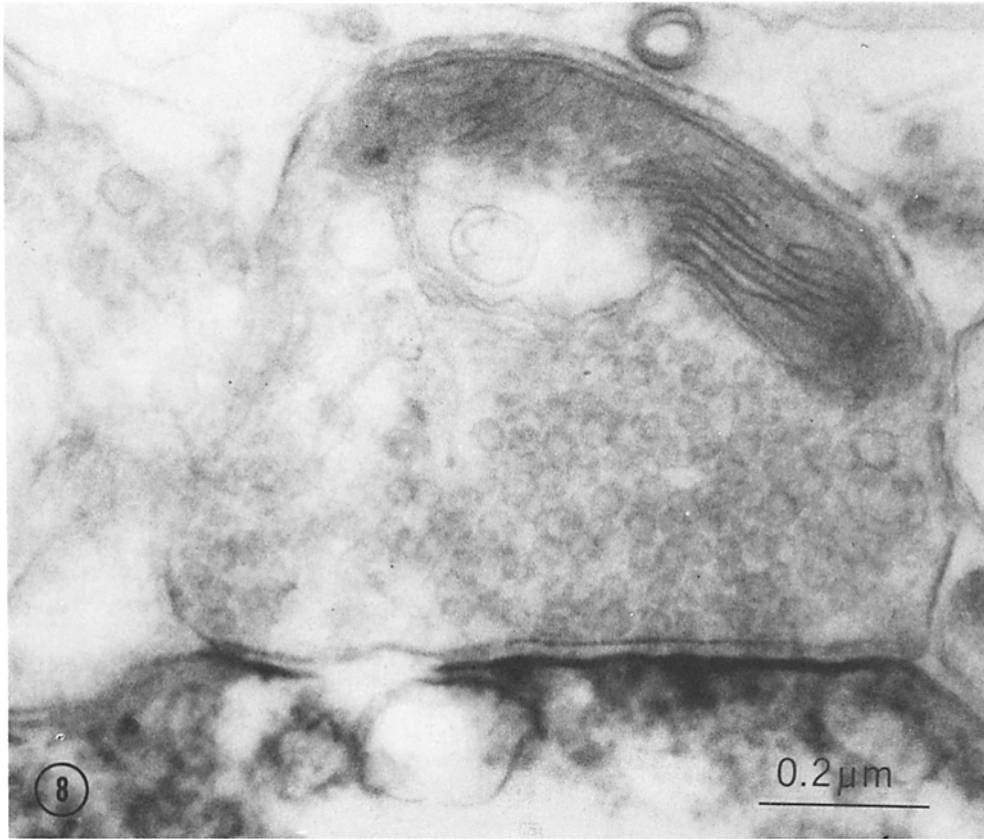


FIGURE 8 Reactive synapse from midbrain. Activity occurs in a thickened postsynaptic band along the region of synaptic contact. $\times 113,000$.

FIGURE 9 Intensely reactive synapse from midbrain. Activity occurs postsynaptically (*Po*) in an irregular layer that includes the postsynaptic membrane and postsynaptic density. In addition, reaction product is localized to the presynaptic membrane (*Pr*). $\times 111,000$.

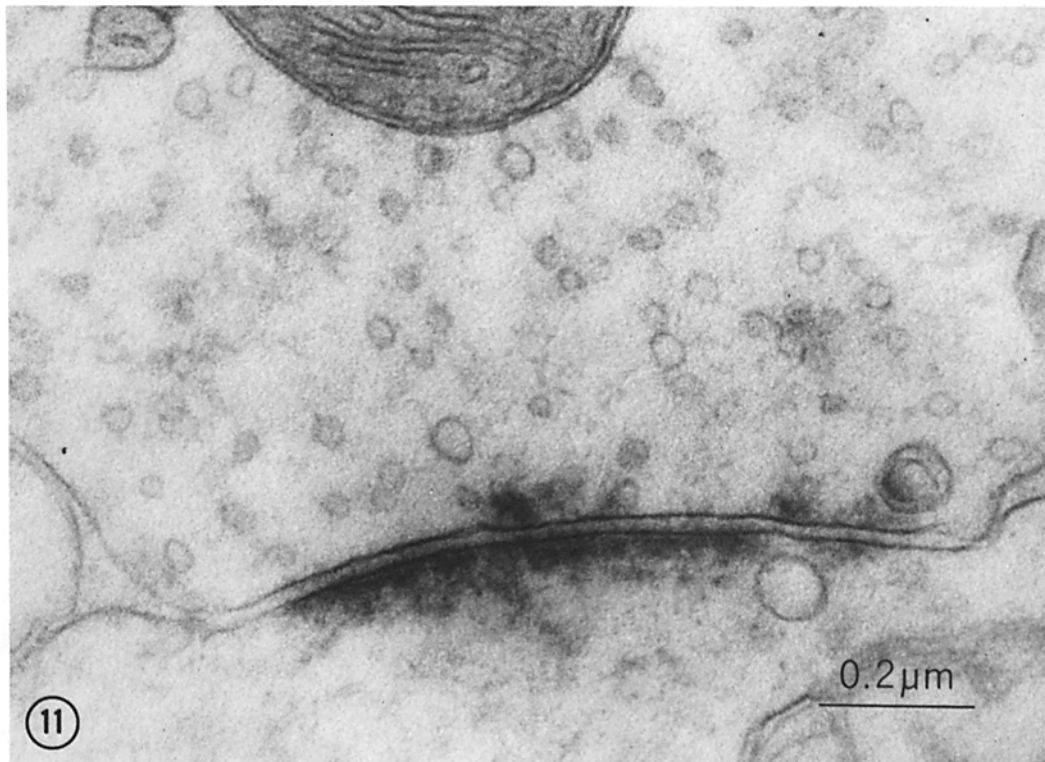
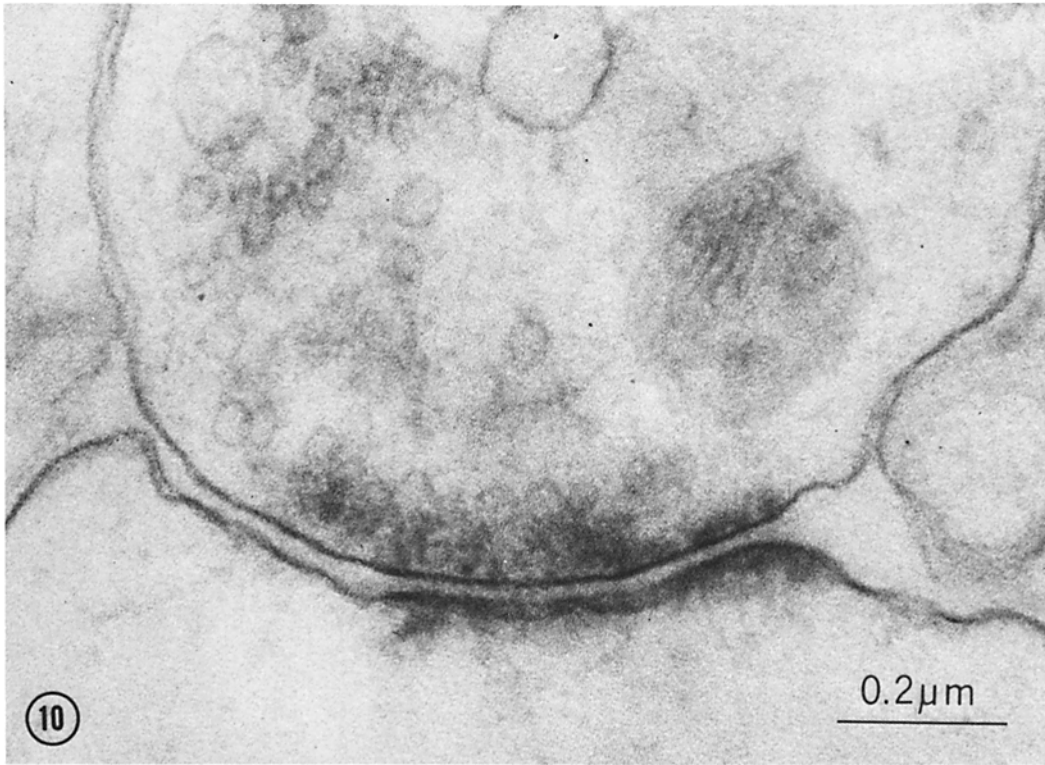


FIGURE 10 Control preparation (midbrain) in which tissue was preincubated in native α -Btx before incubation in the conjugate and reaction for HRP. Reaction product is absent in the pre- and postsynaptic membranes and densities. $\times 112,000$.

FIGURE 11 Control preparation (α -Btx preincubation, midbrain) showing absence of reaction product. Note that membranes in the region of synaptic contact are denser than membranes in other regions. The width of the postsynaptic membrane is less than the thickness of the postsynaptic band of reaction product in reactive synapses. $\times 103,000$.

30). Since some central postsynaptic membranes have membrane granules and some bind conjugated toxin, it is possible that, as in the case of the neuromuscular junction, toxin binding takes place at regions occupied by membrane granules.

Previous studies have indicated that the reticular system (32) and hypothalamic nuclei (33) contain cholinergic neurons or fibers. This study demonstrates the presence of cholinergic synapses in these regions. Reactive synapses were characterized by a predominance of small, clear vesicles while synapses containing many dense-core vesicles (possibly containing biogenic amines) were unreactive. It should be noted, however, that in both synaptosomes and reactive brain regions, only a small proportion of the total number of synapses present were reactive, including those containing clear vesicles. This finding may indicate a relatively small number of nicotinic cholinergic synapses in the central nervous system. Alternatively, lack of staining could be due to a density of receptors below the limits of sensitivity of the technique, or to insufficient penetration of the conjugate.

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