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HISTOLOGICAL LOCALIZATION OF BINDING SITES OF α-BUNGAROTOXIN AND OF ANTIBODIES SPECIFIC TO ACETYLCHOLINE RECEPTOR IN GOLDFISH OPTIC NERVE AND TECTUM

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SUMMARY

Goldfish optic nerve as well as ganglion cell neurites grown in culture selectively bind rhodamine-labeled α-bungarotoxin following tissue fixation. Binding is competed for by unlabeled bungarotoxin, by carbamylcholine and tubocurarine, but not by atropine. In cross-sections, the label is seen confined to axonal bundles. The binding is not detectable without prior fixation and is very faint in brain sections, even after fixation.

To further establish the nature of the binding, immunocytochemical studies were performed, taking advantage of a high cross-reactivity found between goldfish brain and antibodies against eel acetylcholine receptor (AChR). Antigenic sites were detected by an indirect unlabeled antibody complexed to horseradish peroxidase. Anti-AChR antibody binding to optic nerve and neurites in culture correlated with that seen with α-bungarotoxin. Binding of anti-AChR was observed in the brain, and was reduced in the denervated tectum following unilateral optic nerve crush or enucleation.

The results are discussed in relation to functions of receptor proteins in the retinotectal system.

INTRODUCTION

That the optic nerve of the goldfish establishes functional reconnection in the tectum following disruption is well-documented[5,8,26], and the phenomenon has served as the basis of a number of model systems for studies on regeneration and specificity in the nervous system. The finding that α-bungarotoxin (α-butx) is selectively bound within the tectum has led to speculation that acetylcholine (ACh) plays a role in visual

processing. It has also been postulated that cholinergic receptors may play a role in regeneration since, after optic nerve injury in lower vertebrates, there is a rapid loss of binding of α-butx in the tectum followed by a gradual reappearance of binding along with recovered function[2,24]. It has not yet been firmly established whether these brain α-butx binding proteins play a role in neurotransmission and, if so, whether they serve pre- or postsynaptically. One might surmise that the decrease in sites in the tectum following enucleation reflects fiber terminal degeneration and that the α-butx sites that are lost were therefore presynaptic. The number of postsynaptic α-butx receptors might be expected not to change or perhaps to increase[15,27]. Arguments in favor of a postsynaptic assignment for the lost receptors rest on the hypothesis that the optic nerve fibers are cholinergic[3]. It has further been postulated that the putative tectal receptors are involved in cell–cell recognition or in trophic interactions[16,20,25]. Whatever the function of α-butx binding proteins in the goldfish visual system may ultimately prove to be, they may or may not be identical with nicotinic ACh receptors (AChR)[17]. An indication of separate entities is found in reports that α-butx binding in sympathetic ganglia and in cultured sympathetic neurons does not block agonist-induced cholinergic responses[17,20,21]. In the goldfish CNS, however, α-butx binding is reported to block the cholinergic response[25], and recent studies present evidence of a purified α-butx binding protein of the goldfish brain with properties similar to AChR from muscle and from electroplax[18].

In the present study, two histochemical approaches, selective binding of rhodamine-tagged α-butx (R-α-butx) and of antibody to eel electroplax AChR (anti-AChR) used in conjunction with horseradish peroxidase (HRP), are employed to investigate further the nature and location of cholinergic receptor sites in the goldfish visual system.

MATERIALS AND METHODS

Materials

Goldfish (*Carassius auratus*) 6–7 cm in body length obtained from Ozark Fisheries underwent right eye enucleation or intraorbital crush of the right optic nerve as previously described[10].

α-Bungarotoxin was obtained from the Miami Serpentarium. The modified fluorescent-labeled α-butx (R-α-butx) was prepared by the method of Ravdin and Axelrod[22].

Preparation of tissue sections

Optic nerve and brain were removed from adult goldfish, quickly frozen and sectioned in a cryostat. Sections were dried on a hot plate at 70 °C and then fixed for 1 h in AFA, a mixture of 80% aqueous ethanol, formalin and glacial acetic acid (90:5:5, v:v:v).

Explantation

Ten to 14 days after the optic nerve was crushed, the retinas were removed and

cut into 500 μm squares[11]. The retinal explants were placed in culture dishes coated with poly-L-lysine in Leibowitz nutrient medium supplemented with HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.2) and fetal calf serum in the presence of gentamicin sulfate, 5'-FudR (5'-fluorodeoxyuridine) and uridine[11]. After 6–7 days in vitro, cultures were fixed in AFA for 20 min for α-butx-binding studies and for 1 h in the case of immunocytochemical reactions.

Histochemical staining with rhodamine-conjugated α-butx

Following fixation, samples to be tested were rinsed with PB and then incubated for 30 min with 10^{-7} M R-α-butx in PB followed by a 30 min rinse with PB containing 1% BSA. The effect of unlabeled α-butx as well as that of other neuroactive drugs on the binding was studied by preincubation for 30 min followed by rinsing and incubation with R-α-butx. Fluorescence from bound R-α-butx was viewed by epi-illumination on an inverted microscope (Leitz Diavert). The excitation source was a spatially expanded beam from an argon laser (Lexel Model 95-3) at 514.5 nm and 0.5 W. Quantitative measurements of fluorescence intensity were performed by using a thermoelectrically cooled photomultiplier (RCA C31034 A) and photon-counting electronics. An adjustable rectangular diaphragm in the image of the microscope photometer unit (Leitz MPV-1) served to define the field of view.

Binding of anti-acetylcholine receptor antisera (anti-AChR)

Rabbit antisera directed against AChR from eel electric organ was the gift of Professor Sara Fuchs, Weizmann Institute. For localization of AChR antigenic sites, an immunocytochemical method based on unlabeled antibody was used[28]. Fixed tissue sections or explants (full thickness) were first preincubated with normal goat serum (NGS) diluted 1:4 in 0.12 M sodium phosphate buffer, pH 7.2 (PB). After the preincubation the samples were incubated for 1 h with rabbit anti-AChR sera or with preimmune sera at the same dilution. Following this incubation they were rinsed for at least 2 h with PB containing 1% NGS and then incubated for 30 min with goat antirabbit IgG (320 μg/ml, Miles, Elkhart, Ind.). The samples were rinsed for 1 h and then further incubated for 30 min with a solution of 1% NGS containing a soluble complex of peroxidase rabbit anti-peroxidase (3:2), diluted 1:40. After this last incubation the samples were rinsed for 30 min in PB and for an additional 30 min in Tris·HCl buffer (0.05 M, pH 7.6). The bound peroxidase was then reacted with 0.06% hydrogen peroxide and 3,3'-diaminobenzidine, 60 mg/100 ml.

RESULTS

Rhodamine α-butx binding

Explant cultures were fixed after 6 days of growth in vitro and were then incubated with a solution of 10^{-7} M R-α-butx in PB. As can be seen in Fig. 1, R-α-butx binding appeared prominent over neurites and was blocked by preincubation with unlabeled α-butx. Fluorescence intensity measurements indicated that D-tubocurarine and carbamylcholine were effective blockers, while atropine blocked binding only

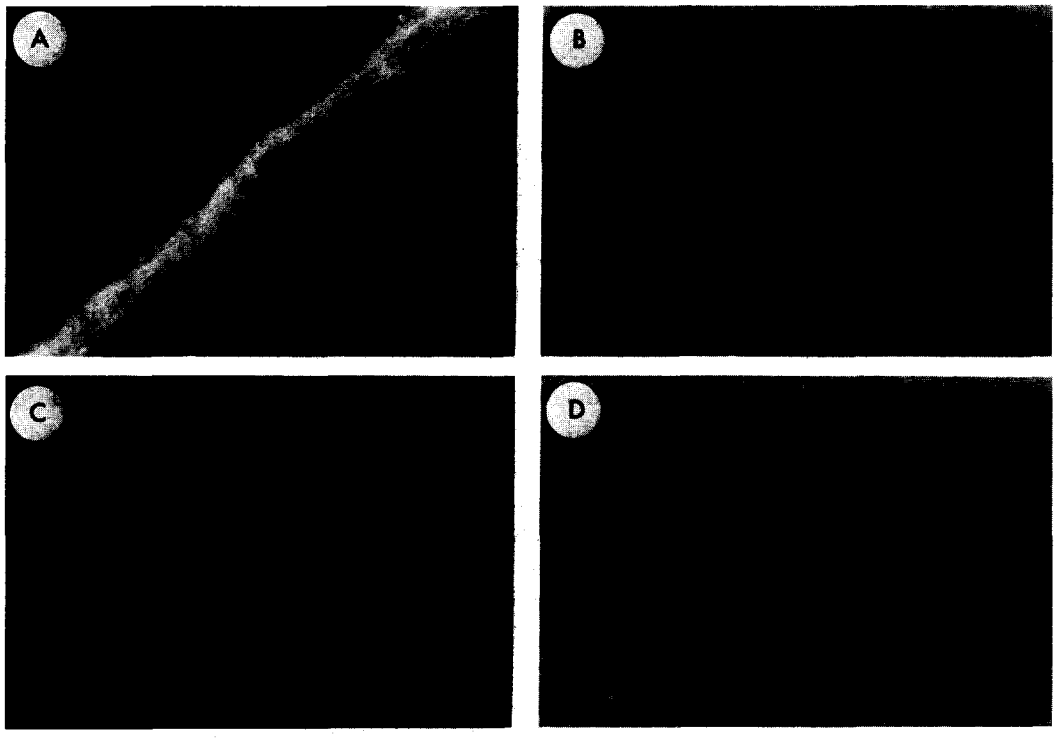

Fig. 1. Binding of rhodamine-α-bungarotoxin to neurites in culture. A: fluorescence micrograph of fixed neurites incubated with 10^{-7} M R-α-butx. B: modulation contrast micrograph[7] of the same region of neurites. C: fluorescence micrograph of neurites preincubated for 30 min with 10^{-6} M unlabeled α-butx followed by 10^{-7} M R-α-butx. D: modulation contrast of the same region of neurites. Space bar = 27 μm.

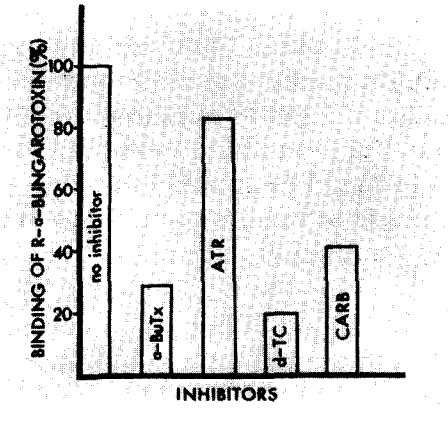

Fig. 2. Effect of neuroactive drugs on the fluorescence intensity of the binding of 10^{-7} M rhodamine-α-bungarotoxin to neurites. Fixed neurites were preincubated for 30 min with the following agents: α-butx (10^{-6} M); ATR, atropine (10^{-3} M); D-TC, D-tubocurarine (10^{-4} M) and CARB, carbamylcholine (10^{-4} M). The average background of fluorescence intensity measured on fixed unlabeled neurites was subtracted from each of the experimental values followed by normalization to set the fluorescence of fixed unblocked R-α-butx neurites at 100%.

TABLE I

Fluorescence intensity of 10^{-7} M rhodamine-α-bungarotoxin binding

For the optic nerve 10 μm sections were used, while for tectum 20 μm sections were used because of the low level of fluorescence in brain. The data presented are normalized to 10 μm. The calculated per cent specific binding is 75 and 29 in nerve and tectum, respectively.

	(A) Optic nerve cross-section	*(B) Optic tectum cross-section*
Unblocked	10.6 ± 3.6*	3.5 ± 0.29
Blocked with 10^{-6} M unlabeled α-butx	2.5 ± 1.5	2.5 ± 0.29

* Each number represents an average of 6–10 measurements of intensity in arbitrary units ± S.E. (see Materials and Methods).

weakly (Fig. 2). The result was confirmed in tissue sections of optic nerve fibers, which exhibited binding as indicated by fluorescence intensity measurements (Table IA). Binding was not observed, however, in tissues that had not previously been fixed, suggesting that in vivo, some of the neurite membrane may not be accessible to the bungarotoxin. Examination of brain cross sections indicated labeling of the optic nerve layer of the tectum. Even after fixation, labeling was faint, compared to that seen in the optic nerve (Table IB).

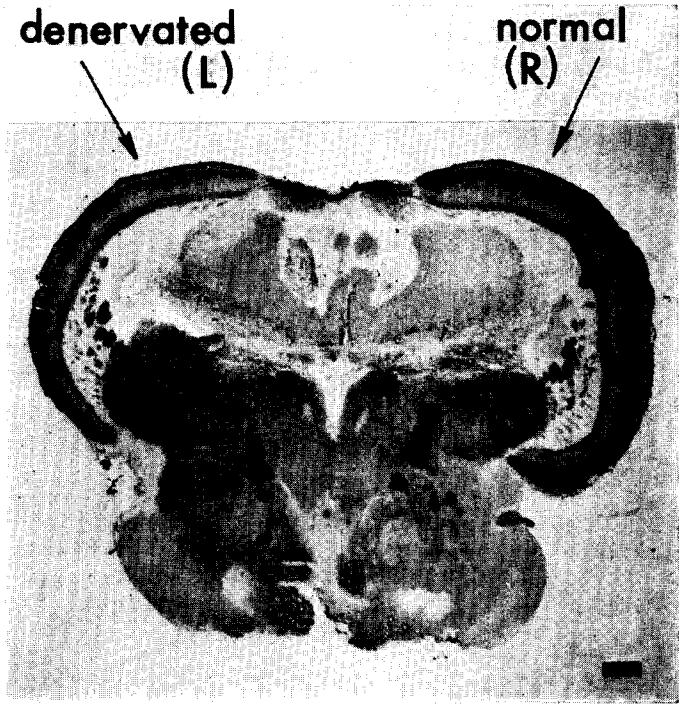

Fig. 3. Effect of eye enucleation of acetylcholine receptor antigenic sites in the tectum. Decreased binding is seen of rabbit anti-AChR sera (diluted 1:40) to the left tectum of goldfish 3 months after enucleation of the right eye. Tectal labeling appears heaviest in the optic nerve fiber layer, but fiber tracts throughout the brain are generally immunoreactive. Space bar = 0.24 mm.

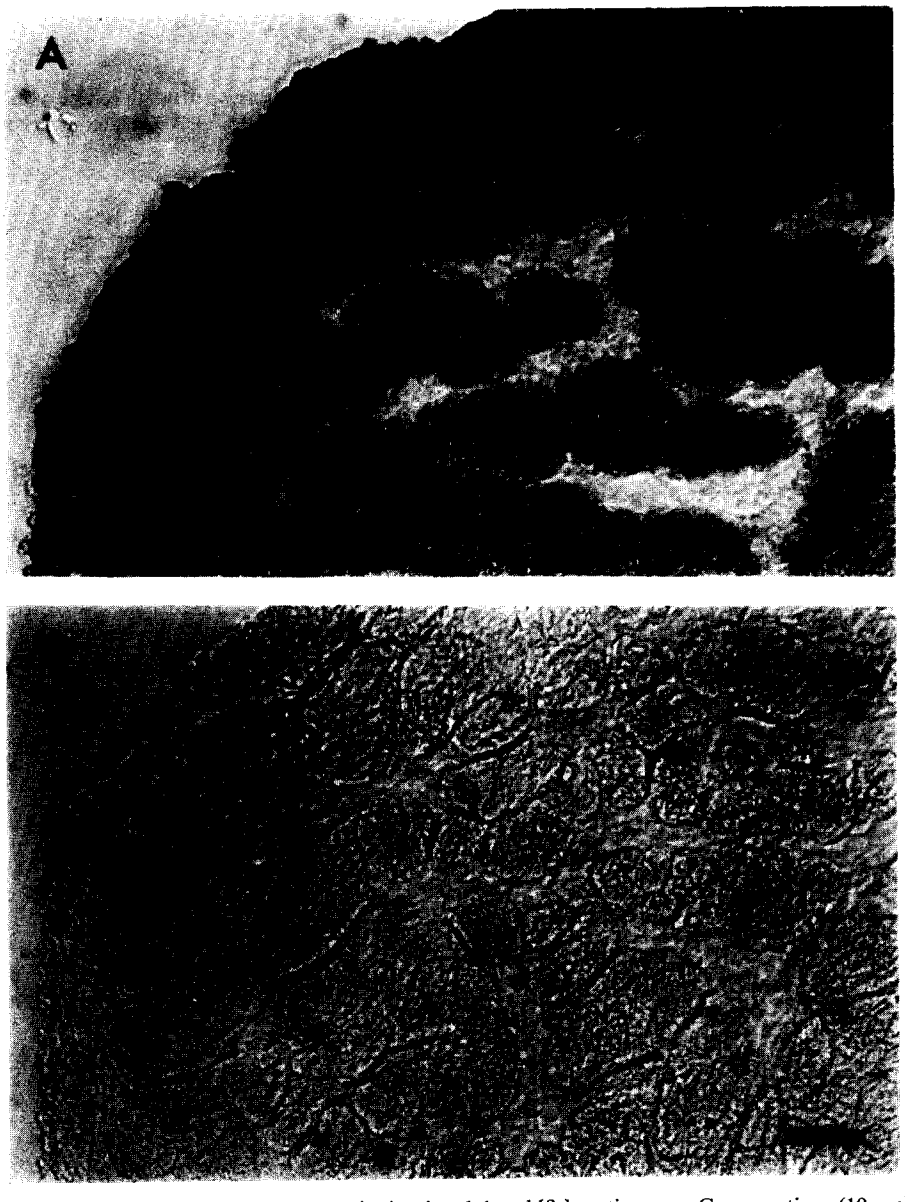

Fig. 4. Localization of AChR antigenic sites in adult goldfish optic nerve. Cross-sections (10 μm) were treated with primary antisera diluted 1:40 followed by goat anti-rabbit IgG and peroxidase–rabbit anti-peroxidase. A: bright-field micrograph of section treated with anti-AChR sera. B: bright-field of section treated as above, except that normal rabbit sera replaced the specific antisera. Space bar = 26 μm.

Anti-AChR binding

Immunological cross-reactivity was established by means of the unlabeled antibody–enzyme immunocytochemical reaction. The anti-eel AChR serum was applied to goldfish brain tectal sections, followed by unlabeled goat anti-rabbit IgG and then the soluble complex of HRP–rabbit anti-HRP. Using this procedure, it could

be demonstrated that the goldfish optic tectum is rich in antigenic sites reactive with the anti-eel AChR serum, up to a dilution of 1:80. No labeling was detected when normal rabbit serum was substituted for the specific antisera, even at a high concentration. The technique was then applied to sections of optic tectum 8–10 days after optic

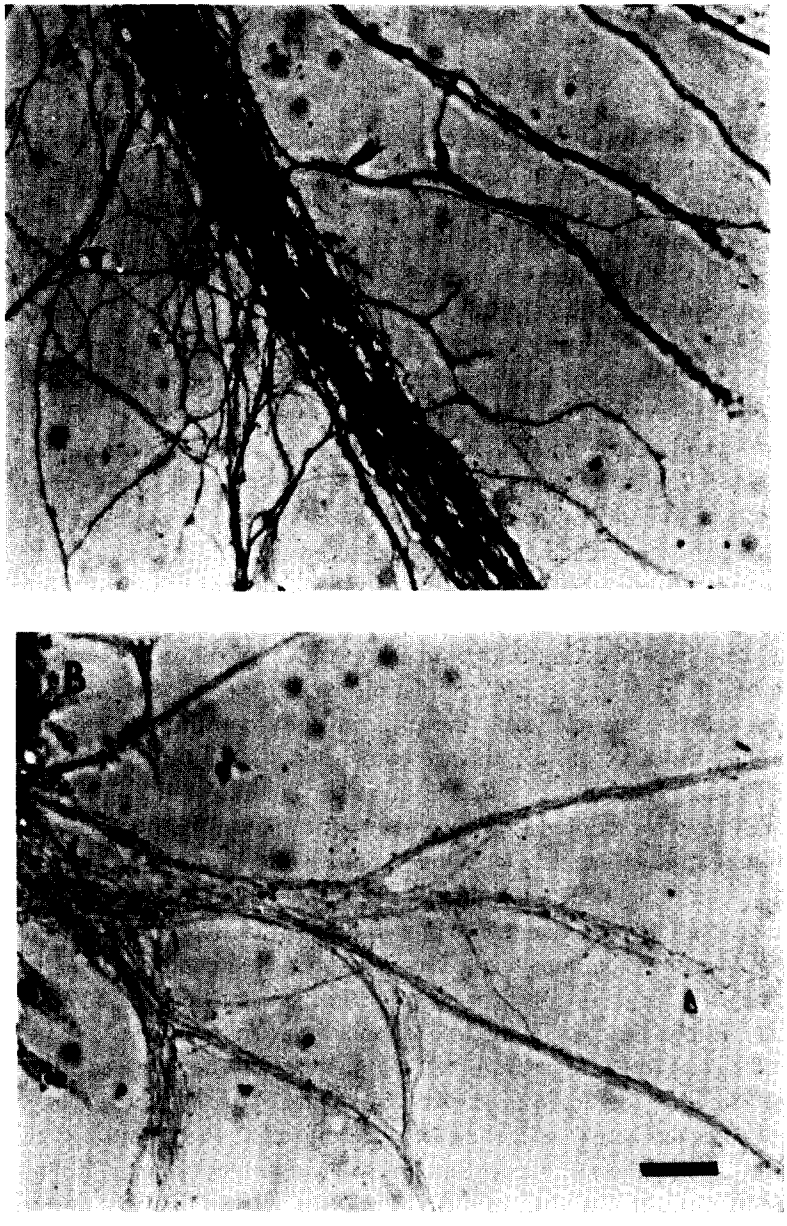

Fig. 5. Localization of AChR antigenic sites on neurites, in vitro. After 7 days in culture, explants were fixed and treated with anti-AChR sera as in Fig. 4. A: bright-field micrograph of neurites treated with anti-AChR, 1:40. B: bright-field micrograph of neurites treated with preimmune sera diluted 1:40. Space bar = 39 μm.

nerve crush or following enucleation (Fig. 3). In each case, a significant loss of AChR binding to the tectum contralateral to the damaged side was clearly demonstrable. Fig. 4 shows selective binding of a 1:40 dilution of antiserum to optic nerve fibers, a result similar to that obtained with R-α-butx. The binding is confined to neuritic fascicles and is absent in the epineurium and surrounding tissue. Dilution up to 1:160 was effective. Binding of anti-AChR was also seen to neurites grown in culture from retinal explants (Fig. 5).

DISCUSSION

Since outgrowing neurites from retinal explants are derived from ganglion cells, the regenerating goldfish visual system in culture affords the possibility of examining optic nerve neurolemmal membrane in the absence of surrounding cells or of presynaptic specializations[10,11]. The finding that rhodamine-labeled α-bungarotoxin binds to neurites and that the binding is competed for by unlabeled toxin as well as by a cholinergic agonist and antagonist suggests the existence of extrasynaptic receptors in the axon. The selective binding seen over fascicles in cross sections of optic nerve verifies the presence of the axonal binding sites in vivo. While α-butx is generally regarded as a binder of synaptic receptors, it has also been reported to bind to invertebrate axons[14] and glia[29], as well as to mammalian dorsal root ganglia[23]. Histochemical evidence for the existence of extrasynaptic binding sites in the present study using R-α-butx was confirmed by means of an immunohistochemical technique, which took advantage of cross reactivity between eel electroplax AChR and goldfish brain. The approach offered a measure of AChR sites independent of possible artifacts associated with the use of labeled α-butx preparations, as discussed below. While there are at present indications supporting both similarities[1] and differences[20,21] between butx and anti-eel AChR binding sites, the correlation in the observed distribution of receptor sites with the two techniques in the present study constitutes supportive evidence for the existence of axonal nicotinic receptor sites.

In our hands, prior fixation is required in order for binding of R-α-butx to be demonstrable. Whether prior fixation is also necessary for anti-AChR binding is not known, since the immunohistochemical technique employed in this study required the procedure. If prior fixation is indeed a requirement for both α-butx and anti-AChR binding, the result would suggest that the axonal binding sites normally lie deeply within the neurolemma or are perhaps located in the axoplasm. A neurolemmal locus would be consistent with a function for nicotinic receptors in neural conduction[13], and while the present findings might be used to support this argument, alternative possibilities should be considered. For example, it is possible that the observed axonal α-butx anti-AChR binding sites are in the process of axonal transport to the synapse and are not physiologically active until they are externalized at the synapse. If this hypothesis is correct, we might also expect that receptors en route to the presynaptic terminals would demonstrate low affinity binding until they were ultimately inserted in their membrane milieu and assumed optimal binding characteristics. It is interesting in this regard that [^{125}I]α-butx binding sites in neurites growing out from symapthetic ganglia are initially hidden and eventually become accessible to the exterior[6].

In reconciling the present results with previous studies in which α-butx derivatives have been used to localize binding sites at the histochemical[19] level or in subcellular fractions[24], the nature of binding affinities must be considered. R-α-butx has up to the present not been successfully employed for histochemical fluorescence localization of nicotinic synaptic binding sites in the nervous system, presumably because of its relatively low sensitivity. In the present studies, the use of a powerful laser source rendered the agent efficacious, but only for a class of sites apparently unavailable prior to fixation. While high affinity sites may have been present in the brain sections, they were presumably not in sufficient local concentration to be detected by fluorescence of the R-α-butx agent. The presence of at least two classes of receptors sites[9,30], i.e. high affinity–low amount at the synapse and low affinity–high amount in axons, could explain both the axonal localization seen in the present study with R-α-butx and reports of synaptic localization seen with more sensitive agents applied at lower concentrations, such as [^{125}I]α-butx[19] and DTAF-α-butx[4]. In comparing results using various α-butx derivatives, it should be borne in mind that derivatization may alter binding specificities[12], and that the native toxin may vary according to source, method of purification, etc.

The amplifying nature of the indirect PAP method proved useful in degeneration studies. It provided sufficient sensitivity for the demonstration of tectal AChR binding sites and of their loss following degeneration of afferent fibers from the retina. [^{125}I]α-butx binding in goldfish tectal sections is also reported decreased following enucleation[19]. The present result is also in general agreement with enucleation studies in goldfish[24], toad and chick[2] brain homogenates in which [^{125}I]α-butx binding to subcellular fractions was observed.

Both the histochemical and in vitro binding approaches indicate that following enucleation, significant tectal receptor binding is retained. That is, after deafferentation, the tectum contains receptors that are *not* in the optic nerve nor in its presynaptic terminals. Whether the surviving receptors are post-synaptic to the degenerating fibers or are in any other tectal neurons (or glia) is not yet established. In any event, it can be concluded that the sites lost following enucleation represent presynaptic terminals or extrasynaptic receptors, or both, following degeneration of the distal stump of the optic nerve. Whether or not this explanation eventually proves correct, the present observation of prominence of AChR in optic nerve supports the suggestion that cholinergic mechanisms are involved in goldfish retinotectal function.

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