RECOVERY OF TECTAL NICOTINIC–CHOLINERGIC RECEPTOR SITES DURING OPTIC NERVE REGENERATION IN GOLDFISH

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SUMMARY

The concentration of cholinergic nicotinic-like sites as measured by alphabungarotoxin (αBuTX) binding, decreased in the goldfish (*Carassius auratus*) optic tectum after optic nerve disconnection. Initially, the rate of loss of sites is greater than the rate of tissue or protein degradation in experiments where disconnection was achieved either by unilateral optic nerve crush or by enucleation of one eye. When the crushed optic nerve is allowed to regenerate and form behaviorally potent connections, the number and concentration of these sites appears restored. Pharmacological studies indicate that the αBuTX binding site in the goldfish optic tectum has a drug binding profile similar to that seen at central or peripheral αBuTX sites in other species.

INTRODUCTION

The regenerative ability of the optic nerve in teleost fish and in amphibia has been well studied by anatomical, electrophysiological and behavioral analysis[1,14,23]. An apparently correct restoration of visual function follows the regeneration of the nerve[7,31]. In addition, there are reported instances of plasticity in the retinotectal system which can be produced by surgical manipulation of either the retina or the tectum[21,30]. To date, few chemical techniques have been applied to elucidate putative chemical mechanisms regulating these phenomena of regeneration, or even to identify the synaptic events that occur during the reconnection process[12]. As a first step in the biochemical analysis of these phenomena, we report alterations in the levels of nicotinic–cholinergic sites in the goldfish optic tectum during regeneration of the optic nerve, as measured with alphabungarotoxin (αBuTX) binding.

[^{125}I]αBuTX has been used as a specific probe for showing the histological localization of cholinergic nicotinic sites[17,26]. Studies with this probe have shown that the central nervous system contains low, but measurable levels of nicotinic receptor sites[27]. Indeed, there is an anatomically heterogeneous distribution of these sites in the mammalian and non-mammalian brain[22,28]. The chick optic lobe has been shown to contain a very high concentration of these sites. The concentration of sites varies during development[15], as well as after enucleation of one eye[4]. Electrophysiological and neurochemical data indicate that αBuTX binding occurs in the toad retinotectal synapses[9,25]. Such evidence indicates that cholinergic sites may be a logical starting point for neurochemical analysis of optic nerve reconnection in goldfish.

Our results show a rapid and substantial loss of αBuTX binding activity after removal of the optic input to the goldfish optic tectum and a restoration of these binding sites during reconnection. The data indicate that the dynamics of synaptic events can be followed during the regenerative process, and suggest that cholinergic sites may be directly involved in the goldfish retinotectal pathway. A preliminary report of some of this data has been previously presented[8].

MATERIALS AND METHODS

Goldfish

Common goldfish (*Carassius auratus*, 10–12 cm) underwent enucleation of one eye or intraorbital optic nerve crush (1 mm behind the eye) after anesthetization by immersion in aqueous Tricaine (Finquel, Ayerst). Visual capacity was judged by positive response to suspended food particles or visually evoked escapes.

Toxin and drugs

Both native αBuTX and [^{125}I]αBuTX were made available by Dr. Jakob Schmidt. The purification and iodination has been previously described[19]. The specific activity of the [^{125}I]αBuTX was 10^6 Ci/mol or lower, depending on the age of the preparation, which typically spanned two half-lives.

Preparation of homogenates and binding assay

Each freshly excised optic tectum was homogenized in 200 μl of buffer containing 10 mM sodium phosphate, 0.4 mM phenylmethylsulfonyl fluoride, 0.013 mM dimethylformamide, 1 mM EDTA, 0.02% sodium azide, at pH 7.4, using a Kontes glass microhomogenizer.

Toxin binding to a particulate fraction was analyzed under conditions of receptor saturation by means of a centrifuge assay whose details have been previously described[29]. Briefly, 90 μl of the homogenate were incubated with 100 fmol of [^{125}I]-αBuTX in a 1.5 ml Eppendorf plastic centrifuge tube in a total volume of 0.3 ml in 10 mM sodium phosphate at pH 7.4. After 3 h at room temperature the incubation was terminated with the addition of 0.8 ml of 0.2 M sodium chloride. Unbound toxin was removed by 3 cycles of centrifugation and washes (1 ml of 0.2 M NaCl) after resuspension. The bound radioactivity was measured after digestion of the washed pellet by

0.2 ml Protosol (New England Nuclear) and counted after the addition of 1.2 ml of 0.4% Permablend in toluene in a liquid scintillation spectrophotometer. In later experiments, the pellets were counted directly in a Beckman Model 8000 gamma counter. In each case, the nonspecific binding was subtracted after it was determined by separate preincubation with a several hundred-fold excess of native toxin. This nonspecific binding was typically about 10% of total binding.

Protein content was determined by the method of Lowry et al.[19] using bovine serum albumin as a standard.

RESULTS

Levels of αBuTX sites in optic tectum after optic nerve crush

In each goldfish tectum there are 30–50 fmol of αBuTX binding sites, the number varying with the size of the tectum, which ranges from 5 to 7 mg and contains approxim-
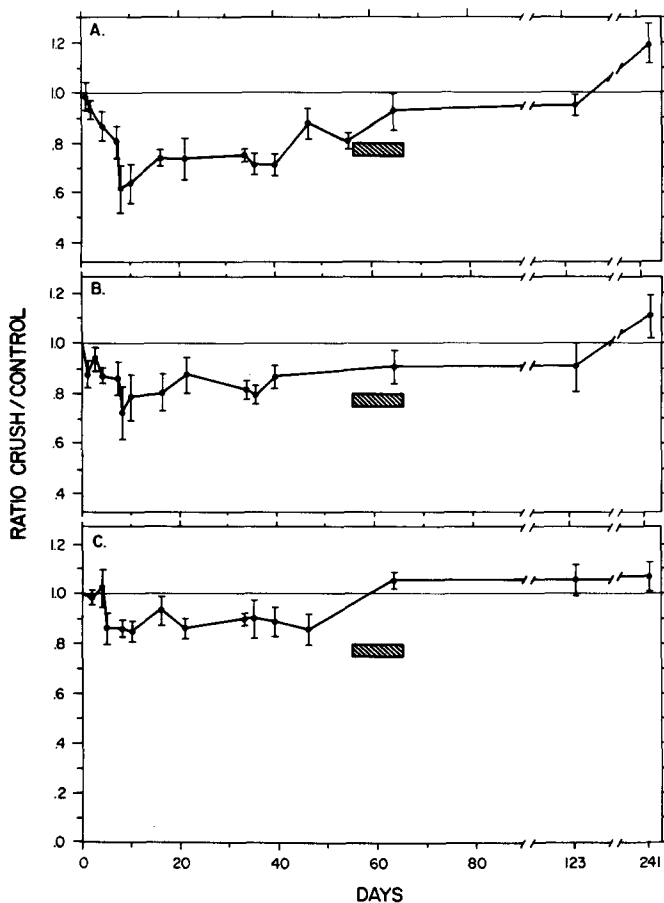

Fig. 1. A: ratio (crush/control) of αBuTX sites per tectum after optic nerve crush. B: ratio (crush/control) of αBuTX sites per mg of tectum after optic nerve crush. C: ratio (crush/control) of tectal tissue weight after optic nerve crush. Shaded areas indicate the period of visual recovery.
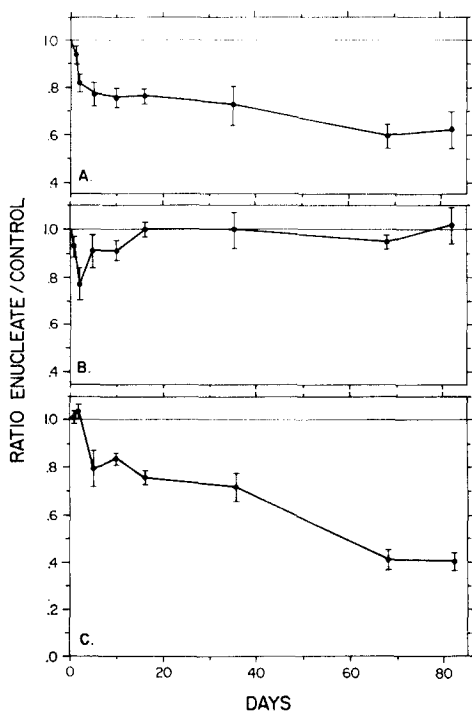
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Fig. 2. A: ratio (enucleate/control) of αBuTX sites per tectum enucleation. B: ratio (enucleate/control) of αBuTX sites per mg of tectum after enucleation. C: ratio (enucleate/control) of tectal tissue weight after enucleation.
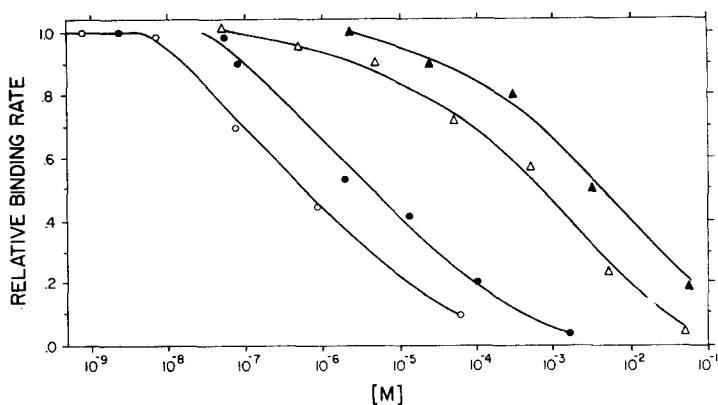

Fig. 3. Inhibition of [^{125}I]αBuTX binding by various drugs; D-tubocurarine (O——O), nicotine (●——●), decamethonium (△——△, and atropine (▲——▲). Between 10–20 intact goldfish tecta were pooled and homogenized (1:40 w/v) in buffer as described in the text. Aliquots of 0.2 ml homogenate were added to equal volumes of buffer containing drugs at various concentrations and incubated for 1 h. Then 300 fmol of [^{125}I]αBuTX (0.1 ml) were added, and binding was allowed to proceed for 20 min. A similar incubation mixture containing 1 μg of cold toxin without drugs was used to determine nonspecific binding. Incubations were then processed as described in the text.

ately 10 % protein. In every case, however, an equal number of sites is present in the left and right tecta in a given goldfish. We therefore, present our data as a ratio of the affected tectum to its control, since the retinotectal projection is completely contra-lateral[24,32]. Each ratio represents the mean ($\pm$ S.E.M.) of at least 3 fish and for data prior to 15 days survival, 6–9 fish.

After optic nerve crush there is an immediate and sharp drop in the total number of tectal αBuTX sensitive sites (Fig. 1) and in the number of sites per mg of tectal tissue. The initial rate of loss in the number of sites is greater than the initial rate of loss of tissue. When demonstrable vision is re-established between 55–65 days, the slopes of both ratios are positive, indicating a continuing recovery of both tissue and sites. This recovery continues after vision returns, even to apparently greater than control levels, at the longest survival intervals studied.

Levels of αBuTX sites in the optic tectum after enucleation of one eye

The curves representing loss of tissue and sites (Fig. 2) are initially similar to those after optic nerve crush. The same sharp and immediate drop is again observed, prior to tissue loss. The ratio of sites per mg of tissue approximates one at later intervals because the rate of tissue degradation parallels that of the loss of sites. That is, although gross tissue degradation continues after 30 days survival, there is no apparent continued loss in αBuTX sites. In separate experiments where binding activity after enucleation was calculated on the basis of total tectal protein, similar results are obtained. The maximal loss in specific binding activity occurred at the 4 day survival point.

Pharmacological characterization of the αBuTX sensitive site

The inhibition of $[^{125}I]\alpha$BuTX binding was investigated in the presence of 4 neuroactive drugs and the K_p (the drug concentration at which the toxin binding rate is reduced to 50 % of its original value) was determined in each case (Fig. 3). Those drugs that have been classified as nicotinic (D-tubocurarine, nicotine and decametho-nium) are effective in the micromolar and lower portion of the millimolar range. The muscarinic drug, atropine, is the least effective and its K_p exceeds the others. Thus, the α-BuTX sensitive site in the goldfish optic tectum shows a similar drug binding profile to that reported for the rat brain[29], and indeed is nicotinic-like.

DISCUSSION

The results show changes in αBuTX binding activity correlated with loss and regrowth of retinal terminals in the goldfish optic tectum. There are several inter-pretations of this data. We suggest the loss reflects changes in tectal cholinergic synapse concentration, and that at least a fraction of the retinotectal terminals are either chol-inergic or cholinoceptive. This conclusion rests on two premises — that the αBuTX binds at nicotinic–cholinergic synapses, and that the losses we obtained are specific to the retinotectal terminals and do not simply reflect general protein degradation or cellular degradation of other cholinergic systems probably present in the tectum[5].

There are anatomical data showing that the αBuTX binding site is synaptic.

Binding is present both pre- and postsynaptically at the neuromuscular junction in several species[17]. Binding is seen at the postsynaptic membrane of rat hypothalamic-derived synaptosomes[16]. Binding of αBuTX has also been observed in the plexiform layers of the retina of several species, including goldfish[34]. Finally, synaptic binding has been shown in the rat hippocampus after in vivo infusion of αBuTX[13]. These suggestive data, together with the demonstration of αBuTX binding to identified synaptosomal fractions[27], indicate that the binding site is probably synaptic at cholinergic sites in the tectum. However, our data does not allow for a distinction between pre- or postsynaptic binding activity.

Although the identity of the retinotectal transmitter or transmitters is not yet established in the goldfish, there is suggestive evidence that glutamic acid may be the principal transmitter in the pigeon, chicken and frog[2,3,10,11,33]. However, more recent reports implicate acetylcholine as the transmitter in the toad retinotectal system[9,25]. Our own data showing a loss of presumptive cholinergic receptor sites suggest that some fraction of the retinotectal terminals may be cholinergic or cholinoceptive in the goldfish. Since postsynaptic membrane specializations have not been reported on goldfish or carp retinotectal terminals[1,21,23], the cholinoceptive alternative is unlikely. That a loss of postsynaptic receptor sites would occur after denervation of the optic nerve is supported by Murray's observation[23] that there were no 'unoccupied' postsynaptic membrane specializations during degeneration of the retinotectal terminals. This suggests that the postsynaptic membranes at retinotectal synapses may be degraded during degeneration. A decline in tectal synaptic density has also been reported after denervation in the toad optic tectum as well as in the carp[20,24].

The rapid loss of αBuTX binding activity obtained in the initial days after denervation would apparently rule out secondary degeneration of cholinergic cells probably present in the tectum[5]. Indeed, only a fraction of the total tectal αBuTX binding sites are lost after denervation, and this loss does not continue with further protein and tissue degradation at later survival intervals. Finally, the rate of decay of αBuTX binding activity closely approximates the rate of degeneration of the retinotectal terminals in the fish and toad[23,24]. Thus, our interpretation is that there is a *primary loss of cholinergic synapses* after denervation, followed by a gradual recovery which continues even after vision is reestablished.

ACKNOWLEDGEMENTS

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