Two Distinct (−)Nicotine Binding Sites in Goldfish Brain

IDENTIFICATION AND CHARACTERIZATION OF PUTATIVE NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR SUBTYPES*

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The binding of (−)-[3H]nicotine to membrane fragments and a detergent solubilized fraction of goldfish brain was characterized. (−)-[3H]nicotine binding was not displaced by α-bungarotoxin, but was displaced by (−)-nicotine and carbamoylcholine with Kᵢ of approximately 8.6 and 86 nM, respectively. Preincubation of solubilized membrane extract with α-bungarotoxin-coupled Sepharose resulted in the loss of approximately 50% of the (−)-[3H]nicotine binding protein from the eluent and an increase in (−)-[3H]nicotine binding to the gel compared to control, non-α-bungarotoxin Sepharose. 125I-α-bungarotoxin binding protein in the eluent from the same preincubation experiments was totally removed. In addition, incubation of the solubilized tissue extracts with α-bungarotoxin-coupled Sepharose resulted in an increase in the affinity for (−)-[3H]nicotine in the eluent (mean Kᵢ = 3.1) compared to control solubilized tissue extracts (Kᵢ = 6.4 nM). Specific (−)-[3H]nicotine binding sites could be eluted from the α-bungarotoxin-coupled Sepharose with carbamoylcholine and D-tubocurarine. Similar to previously reported 125I-α-bungarotoxin binding data, eye removal resulted in an approximately 40% decrease in (−)-[3H]nicotine binding in the contralateral tectum compared to that in the ipsilateral tectum. These data indicate that at least two distinct subtypes of (−)nicotine binding sites may be present in goldfish brain, one which binds α-bungarotoxin and (−)nicotine and another which binds only (−)nicotine.

Biochemical characterization of the neuronal nicotinic acetylcholine receptor (nAChR) has progressed slowly compared to the nAChRs present in the electroplaque of electric fish or the vertebrate neuromuscular junction for two main reasons: 1) the receptor density in the central nervous system (CNS) is lower than that in the other two tissue types and 2) investigations have been hampered by a lack of suitable ligands. A number of reports have suggested that in some higher vertebrate species, the α-bungarotoxin (α-Bgt) binding protein in neuronal tissue may not be a nAChR, although a role for the α-Bgt binding protein in cholinergic pathways can not yet be ruled out in these systems (Brown and Fumigalli, 1977; Carbonetto et al., 1978; Ravdin and Berg, 1979). In goldfish, toad, and frog, however, a considerable body of evidence exists to suggest that at least some of the protein recognized by α-Bgt may be functional neuronal nAChR. The α-Bgt binding protein in goldfish (Carassius auratus) optic tectum has been extensively studied and resembles electroplaque and muscle nAChRs in some of its pharmacological, hydrodynamic, and metabolic properties (Oswald and Freeman, 1979, 1981). Approximately half of the α-Bgt binding sites are associated with the retinotectal projection (Schechter et al., 1979, Oswald et al., 1980) and the majority of α-Bgt binding is concentrated in those tectal layers possessing primary retinotectal synapses. The same tectal layers also contain high concentrations of choline acetyltransferase (Tumosa and Stell, 1986) and acetylcholinesterase (Oswald et al., 1980). In addition, α-Bgt inhibits synaptic transmission (Marshall, 1981), and injection of nanomolar concentrations of α-Bgt has been demonstrated to block visual responses in toad (Freeman, 1977) and goldfish optic tectum (Schmidt and Freeman, 1980; Freeman et al., 1980).

More recently, a subset of monoclonal antibodies which interact with the main immunogenic region of Electrophorus electroplaque nAChR have been shown to react with the α-Bgt binding protein in frog (Sargent et al., 1984) and goldfish brain (Henley et al., 1986a) with high affinity. These antibodies were found to label the same areas of the optic tectum as 125I-α-Bgt, suggesting shared antigenic determinants between the electroplaque nAChR and goldfish and frog CNS α-Bgt binding protein. One of these monoclonal antibodies (monoclonal antibody 47) was used to demonstrate that an α-Bgt binding protein, biosynthetically labeled with [35S]methionine following injection of the isotope into the eye, is synthesized in the retina and transported to the optic tectum via the optic nerve (Henley et al., 1986b). It has been proposed that the α-Bgt binding protein in the goldfish retinotectal system may be important for the formation and maintenance of synaptic connections in the goldfish optic tectum (Freeman, 1977; Schmidt, 1979).

The α-Bgt binding protein from the CNS of the insect Locusta migratoria has also been well characterized (Breer et al., 1985). The purified receptor protein migrates as a single band on polyacrylamide electrophoresis under both native (Mᵢ = 250,000–300,000) and denaturing conditions (Mᵢ = 65,000). The isolated receptor subunits cross-react with some monoclonal antibodies raised against the nAChR from Torpedo marmorata. These workers propose that the α-Bgt binding protein from locust CNS is an oligomeric complex composed of four identical subunits and may represent an ancient prototype form of an ancestral nAChR. This suggestion is supported by the subsequent reconstitution of the purified protein into planar lipid bilayers where, using patch clamp techniques, it has been shown that this α-Bgt binding

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† The abbreviations used are: nAChR, nicotinic acetylcholine receptor; Ach, acetylcholine; α-Bgt, α-bungarotoxin; MOPS, 3-(N-morpholino)propanesulfonic acid; CNS, central nervous system.

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protein is a functional nACHR (Hanke and Breer, 1986).

[1H]Acetylcholine (Ach), in the presence of atropine, and [1H]nicotine have been proposed as specific ligands for neuronal nACHRs (Romano and Goldstein, 1980; Marks and Collins, 1982; Schwartz et al., 1982). [1H]Ach (in the presence of atropine) seems to bind to one high affinity site in rat brain with an average KD of 12.3 nM (Schwartz et al., 1982). Studies with racemic mixtures of [1H]nicotine have suggested the presence of two binding sites (Romano and Goldstein, 1980). However, Schwartz and co-workers (1982) demonstrated that (+)-nicotine, rather than (-)-nicotine, is a far more potent antagonist of [1H]Ach binding. Subsequently, (-)-[1H]nicotine has been shown to bind to one high affinity site in rat brain and this site appears to be identical to the [1H]Ach binding sites (Martino et al., 1985; Lippielio and Fernandes, 1986). In addition, both [1H]nicotine and [1H]Ach binding sites in rat brain are similarly decreased by chronic administration of cholinesterase inhibitors (Schwartz and Kellar, 1983; Costa and Murphy, 1983) and are similarly sensitive to disulfide bond degradation (Martino et al., 1986).

Whiting and Lindstrom (1986) have purified a protein from chick brain using a monoclonal antibody raised against the main immunoreactive region of Electrophorus electricus electric organ nACHRs. This protein is capable of binding (-)-[1H]nicotine but not (-)-[1H]a-Bgt. Taken together these results suggest that (-)-[1H]nicotine may be a useful ligand for studying neuronal nACHRs. The recent demonstration by Goldman et al. (1986) that mRNA species with homologies to both mouse and the PC12 rat pheochromocytoma cell line nACHR exist in rat brain suggests that more than one type of neuronal nACHR may be present in the CNS. One attractive possibility is that (-)-nicotine and a-Bgt are capable of interacting differentially with nACHR subtypes.

The aim of this study was to characterize (-)-[1H]nicotine binding protein in goldfish brain and determine its relationship to the 125I-alpha-Bgt binding protein. We provide evidence for the existence of at least two populations of (-)-nicotine binding sites, one of which is associated with the a-Bgt binding protein and one which is not.

**EXPERIMENTAL PROCEDURES**

**Preparation of Membranes and Detergent Extracts** — The brain was removed from ice-anesthetized goldfish immediately before use. The wet weight of tissue was determined in a cooled glass homogenizer to 3-5 ml of ice-cold assay buffer composed of either (a) 120 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 50 mM Tris, pH 7.4, (b) 50 mM MOPS, 1 mM EDTA, pH 7.4, or (c) 50 mM MOPS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 1 mM D-tubocurarine, 0.1 mM phenylmethylsulfonyl fluoride. The eluent was dialyzed against 1 mM MOPS, 1 mM EDTA buffer containing 0.1% Triton X-100 at 4°C overnight to remove the D-tubocurarine and carbamoylcholine.

**Choline-Cardboxymethyl Affinity Gel—** Choline-carboxymethyl affinity gel was prepared as described by Reynolds and Karlin (1978). Experiments performed using the choline-carboxymethyl affinity gel were performed exactly as described for a-bungarotoxin-coupled Sepharose.

**(-)-1H Nicotine Binding Assay—** The filter assay procedure used was essentially the same for both membrane fragments and solubilized tissue preparations. (-)-[1H]Nicotine (typically at 10 nM, final concentration) was added to disposable 5-ml glass test tubes containing either ice-cold MOPS-EDTA or MOPS-EDTA and carbamoylcholine (to a final concentration of 20 mM) and tissue, usually at a final concentration of 20 mg wet weight/assay. (-)-[1H]Nicotine was then added and the tubes incubated on ice in the dark for 1 h. The reaction mixtures were filtered under negative pressure on 0.65-mm (membranes) or 0.2% (solubilized tissue preparations) polyvinyl alcohol prewetted Whattman GF/C glass filters (Brown et al., 1983). The filters were washed rapidly three times with 4 ml of ice-cold MOPS-EDTA and assayed for radioactivity.

To determine the (-)-[1H]nicotine binding activity retained on the Sepharose, aliquots of the diluted, well suspended gel and the appropriate concentration of (-)-[1H]nicotine were added to tubes with or without 100 nM carbamoylcholine. The tubes were then caged and mixed by inversion for 1 h at 4°C. Following incubation (-)-[1H]nicotine binding activity was assessed using the filtration assay described above.

**D-Tubocurarine Binding—** Equilibrium binding of 125I-alpha-Bgt to Triton X-100-solubilized brain extracts was determined as previously described (Oswald and Freeman, 1981). Briefly, tissue was incubated in the presence or absence of 1 mM carbamoylcholine for 1 h at room temperature with 2 nM 125I-alpha-Bgt. Protein in the solubilized tissue extract was then precipitated by addition of 33% saturated ammonium sulfate at room temperature and rapidly filtered through Milipore EHWP or GVWP filters. The filters were then washed three times with 4-ml aliquots of ammonium sulfate and allowed to dry, and the radioactivity was subsequently determined. The KD and Bmax were determined using a nonlinear least squares fit to the data as described previously (Oswald and Freeman, 1979).

The initial rate of 125I-alpha-Bgt binding was measured essentially as for equilibrium binding. Preliminary time course experiments were carried out to establish that the rate of binding remained linear for the first 1.5-2 min. Triton X-100-solubilized tissue extracts were then incubated with 200 nM 125I-alpha-Bgt in the presence or absence of varying concentrations of carbamoylcholine or (-)-nicotine at room temperature for 1 min and then rapidly filtered as described.

**Sucrose Density Gradients—** Sucrose density gradients (5-20% continuous sucrose gradients in MOPS-EDTA, 1% Triton X-100 buffer, pH 7.4) were prepared previously (Horne et al., 1986). Aliquots (1 ml) of solubilized tissue extract containing 1% sucrose and catalase were layered on top of the gradient. The tubes were filled with buffer and sealed, and the gradients were centrifuged for 2 h at 45,000 rpm in a Beckman VTi50 vertical rotor. Fractions (0.6 ml) were collected from the bottom of the tube and aliquots (200 µl) from alternate fractions from the same gradient assayed for specific 125I-alpha-Bgt and (-)-[1H]nicotine binding. Control experiments had
previously determined that the sucrose concentrations included in the assay samples did not affect the ligand binding characteristics.

**Materials**—Goldfish were obtained from Grassylfork Fisheries, Martinsville, IN. All the reagents used were of analytical grade.

Protease inhibitors, (−)-nicotine, and cyanogen bromide activated Sepharose beads were obtained from Sigma. (−)-[3H]nicotine (specific activity 60.4 Ci mmol⁻¹) and 125I-α-Bgt (70–140 Ci mmol⁻¹) were from New England Nuclear. α-Bungarotoxin was prepared from crude lyophilized *Bungarus multicinctus* venom (Sigma) by the method previously described by Freeman et al. (1980) except that MonoQ and Superose 12 columns were used in conjunction with a Pharmacia FPLC.

**RESULTS**

**Equilibrium Binding of (−)-[3H]Nicotine**

The binding of (−)-[3H]nicotine to membrane fragments and detergent-solubilized tissue preparations is shown in Fig. 1. The *K*ₐ values were 4.89 ± 0.34 and 6.39 ± 0.9 nM and the *Bₐₙₐₗ* values 4.71 ± 0.12 and 2.1 ± 0.27 fmol/mg wet weight tissue, respectively (mean ± S.E. of three to six separate experiments each with tissue from three to seven goldfish). Solubilization of the tissue resulted in a reduction in the apparent number of binding sites by approximately 50%. These findings may reflect incomplete solubilization or detergent inactivation of the binding sites by Triton X-100 under the regime used. Inclusion of protease inhibitors did not alter the number of (−)-[3H]nicotine binding sites in the solubilized tissue extract.

The effects of (−)-[3H]nicotine binding to solubilized membrane extracts of increasing concentrations of (−)-nicotine, carbamoylcholine, and α-Bgt are shown in Fig. 2A. Both (−)-nicotine and carbamoylcholine displaced (−)-[3H]nicotine with *K*ₐ values of approximately 8.6 and 86 nM, respectively, whereas α-Bgt had no notable effect over the concentration range investigated. The *K*ₐ values were calculated from the *IC₅₀* using the method described by Cheng and Prusoff (1973). The results suggest that α-Bgt binds to a separate site(s) from (−)-[3H]nicotine and that its occupation of this site(s) does not appear to modify (−)-[3H]nicotine binding. These data can be explained by the existence of separate (−)-[3H]nicotine and 125I-α-Bgt binding protein moieties or to spatially and functionally distinct sites on the same protein where occupancy of the α-Bgt binding site(s) does not influence the binding of (−)-[3H]nicotine.

**Binding of 125I-α-Bgt**

Equilibrium binding studies of 125I-α-Bgt to Triton X-100-solubilized membrane extracts yielded a *K*ₐ of 0.23 ± 0.06 nM (n = 4) and were in good agreement with previously published results (Oswood and Freeman, 1981). Comparison of 125I-α-Bgt to (−)-[3H]nicotine binding in this study show that there are approximately the same number of binding sites for each ligand, approximately 1.5–2 fmol/mg wet weight of tissue. To determine the effects of carbamoylcholine and (−)-nicotine on 125I-α-Bgt binding, initial rate studies were performed. The initial rate of binding was investigated to avoid complications caused by the high affinity and slow off rate of 125I-α-Bgt binding. As shown in Fig. 2B, both compounds reduced 125I-α-Bgt binding in a dose-dependent manner but were half maximally effective at concentrations approximately 2 orders of magnitude higher than were necessary to displace (−)-[3H]nicotine binding. The *K*ₐ values were approximately 0.16 and 5.35 μM for (−)-nicotine and carbamoylcholine, respectively, as compared to approximately 8.6 and 86 nM, respectively, for (−)-[3H]nicotine binding (see Fig. 2A). Another difference observed was that substance P which has been demonstrated to displace 125I-α-Bgt binding to *Torpedo* and BC3H1 cell membranes also reduced 125I-α-Bgt binding to the Triton X-100-solubilized goldfish brain membrane extracts used in this study but did not affect (−)-[3H]nicotine binding. These data suggest that (−)-[3H]nicotine and 125I-α-Bgt bind to different sites but that the 125I-α-Bgt binding site is recognized and/or modulated by cholinergic ligands whereas the (−)-[3H]nicotine binding site is not recognized or modulated by α-Bgt.

Fig. 1. Saturation binding curves and Scatchard plots of (−)-[3H]nicotine to membrane and Triton X-100-solubilized tissue preparations from goldfish whole brain. Samples of 10–20 mg wet weight of tissue were incubated with the indicated concentrations of (−)-[3H]nicotine in either the presence (nonspecific) or absence (total binding) of 100 μM carbamoylcholine. The difference represents the specific binding which is plotted. Scatchard plots of the specific binding data are shown in the insets. A, goldfish whole brain membranes; B, goldfish whole brain Triton X-100 extract. The *K*ₐ and *Rₐₙₐₗ* values were 4.89 ± 0.34 nM and 4.71 ± 0.12 fmol/mg wet weight tissue and 6.39 ± 0.09 nM and 2.1 ± 0.27 fmol/mg wet weight tissue for the membrane fragments and Triton X-100-solubilized extract, respectively. The values given are the mean ± S.E. from four separate experiments for membrane fragments and nine separate experiments for solubilized brain extracts, each starting with 1–5 g of brain pooled from six to ten fish.
Effects of Eye Removal on (-)-[3H]Nicotine Binding

Eye removal in goldfish has previously been shown to result in a 30-40% decrease in the number of [125I]-α-Bgt binding sites in the contralateral tectum compared to the ipsilateral tectum (Schechter et al., 1979; Oswald et al., 1980; Henley et al., 1986b). A quantitatively similar loss occurs in the number of (-)-[3H]nicotine binding sites with a 54% ± 10% (n = 3 with four to seven fish used/experiment) reduction in (-)-[3H]nicotine binding sites in the contralateral tectum compared to the control ipsilateral tectum. These data suggest that the (-)-[3H]nicotine and 125I-α-Bgt binding sites appear to be similarly regulated and may, at least partially, be colocalized in the same neuronal elements, possibly on the same protein.

Comparison of (-)-[3H]Nicotine and 125I-α-Bgt Binding Proteins

Hydrodynamic Properties—To determine if the (-)-[3H]nicotine and 125I-α-Bgt binding fraction(s) of solubilized brain could be separated by their hydrodynamic properties, Triton X-100 brain extracts were investigated by sucrose density gradient centrifugation (data not shown). The binding proteins for the two ligands sedimented at 11.4 S (see Oswald and Freeman, 1979) and were not separable within the resolution of this technique. These findings are similar to those previously reported by Schneider et al. (1985) in chick optic lobe. The data suggest that either the binding sites for the two ligands reside on the same protein moiety or on two different proteins which have similar hydrodynamic properties.

α-Bgt-coupled Sepharose—To define more precisely whether the (-)-[3H]nicotine and the 125I-α-Bgt binding sites reside on the same or different protein(s), a series of experiments were performed with α-Bgt-coupled Sepharose. Both high specific activity (see Fig. 3) and normal α-Bgt-coupled Sepharose (data not shown) were used. Qualitatively similar results were obtained from both types of gel. The use of high specific activity α-Bgt-coupled Sepharose, however, required a smaller volume of gel to deplete completely the solubilized brain extract of 125I-α-Bgt binding protein. Nonspecific binding of the (-)-[3H]nicotine binding protein to the gel was therefore minimized.

Triton X-100-solubilized brain extract was incubated with varying proportions of high specific activity α-Bgt-coupled and control (non-α-Bgt) Sepharose. The total volume of gel was kept constant in all the conditions (64 μl of gel/1 ml of brain extract). Following incubation, the eluent was collected and aliquots incubated with 10 nM (-)-[3H]nicotine of 2 nM 125I-α-Bgt. As the proportion of α-Bgt-coupled gel to control gel was increased, the amount of 125I-α-Bgt binding protein remaining in the eluent was correspondingly reduced and eventually completely removed. (-)-[3H]Nicotine binding protein in the eluent was also reduced in a dose-dependent fashion, but at concentrations of α-Bgt-coupled Sepharose equal to or greater than those which totally removed 125I-α-Bgt binding protein, approximately 50% of the (-)-[3H]nicotine binding protein remained in the eluent. No differences were observed in the total protein concentration present in the eluent between control and α-Bgt-coupled Sepharose incubation conditions (determined by the method described by Bradford, 1976). Similar results were observed in both the presence and absence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM 1,10 phenanthrolene, 1 mM iodoacetamide, 1 μM pepstatin A, 1 mg/liter antipain, 1 mg/liter leupeptin). Inclusion of 1 μM α-Bgt in the incubation mixture prevented the retention of (-)-[3H]nicotine binding protein on the α-Bgt-coupled Sepharose. These data suggest that a portion of the (-)-[3H]nicotine binding sites can be removed from the extract by α-Bgt-coupled affinity Sepharose by a mechanism which involves a specific α-Bgt recognition site on the (-)-[3H]nicotine binding protein.

To investigate observation further, the binding of (-)-[3H]nicotine and 125I-α-Bgt to control and α-Bgt-coupled Sepharose was also investigated. Following incubation with solubilized brain extract, the control or α-Bgt-coupled Sepharose was pelleted by centrifugation and the supernatant removed and saved. The Sepharose was washed by centrifugation with 1 ml of MOPS-EDTA then 1 ml of 0.5 M NaCl in MOPS-EDTA and finally with 1 ml of MOPS-EDTA (all at 0°C). The resultant Sepharose pellet was resuspended and aliquoted for binding assays as described. Control experiments were performed to confirm that neither (-)-[3H]nicotine nor 125I-α-Bgt bound to α-Bgt-coupled or control Sepharose which had not been preincubated with solubilized tissue extract. The concentration of (-)-[3H]nicotine and 125I-α-Bgt binding protein immobilized on the washed toxin and control Sepharose was then determined. Similarly, the amount of binding activity which remained in the solubilized brain extract following incubation with the toxin or control Sepharose was also assayed (see Table I).

Approximately 10-fold more (-)-[3H]nicotine and 125I-α-Bgt binding sites were retained on α-Bgt-coupled Sepharose than on control Sepharose. In total (the sum of the sites present in the eluent and on the Sepharose), the control Sepharose incubation conditions possessed approximately twice as many detectable 125I-α-Bgt binding sites than those present in the α-Bgt-coupled Sepharose incubation conditions. This may be explained by the existence of multiple binding sites for α-Bgt on at least a portion of the α-Bgt binding proteins as has been observed previously in Torpedo electroplaques (for review see Karlin, 1980) and rat brain α-
The values given are the mean ± S.E. of four to seven separate experiments and are expressed as fmol of binding protein present in each assay. The concentration of (-)-[3H]nicotine or 125I-α-Bgt used for each assay was 10 or 2 μM, respectively. At these ligand concentrations, the (-)nicotine binding sites were approximately 63% saturated and the α-Bgt binding sites were fully saturated. The values given below have been corrected to account for this difference in the fraction of binding sites occupied.

| Gel | Eluent | Gel | Eluent |
|-----|--------|-----|--------|
| (-) [3H]Nicotine | 11.04 ± 1.16 | 8.22 ± 1.49 | 15.3 ± 0.93 | 17.67 ± 2.58 |
| 125I-α-Bgt | 13.75 ± 3.02 | 1.92 ± 0.91 | 1.62 ± 1.05 | 25.83 ± 2.96 |

Fig. 4. (-)[3H]Nicotine binding protein retained by α-Bgt-coupled Sepharose. Solubilized brain extract (1 ml) was preincubated with a variety of ligands for 30 min at room temperature prior to incubation with 150 μl of α-Bgt-coupled or control Sepharose (5 h with rotation at room temperature). The Sepharose was washed three times by centrifugation and the amount of (-)-[3H]nicotine binding to the gel assessed. The results are expressed in fmol of specific binding retained by the Sepharose. The values are the mean ± S.E. of 4-5 determinations. The conditions shown are: control Sepharose (A), α-Bgt-coupled Sepharose (B), α-Bgt-coupled Sepharose with 10 mM carbacholcholine (C), α-Bgt-coupled Sepharose with 10 μM (-)nicotine (D), α-Bgt-coupled Sepharose with 3 μM α-Bgt (E), and α-Bgt-coupled Sepharose with no tissue (F).

Bgt binding protein (Kemp et al., 1985). The total number of (-)-[3H]nicotine binding sites were similar in both the control and α-Bgt-coupled Sepharose conditions. These data suggest that if multiple (-)-[3H]nicotine binding sites are present on these proteins they are not obstructed by binding to the immobilized α-Bgt.

A number of additional control experiments were carried out to ensure that the α-Bgt-coupled Sepharose did not act as an ion exchange resin. Inclusion of either 10 mM carbacholcholine, 10 μM (-)nicotine, or 3 μM α-Bgt with the detergent extract and the α-Bgt-coupled Sepharose reduced (-)-[3H]nicotine binding to the α-Bgt-coupled Sepharose to levels similar to those retained by control Sepharose (see Fig. 4). These data confirm the eluent binding observations.

To demonstrate conclusively that the retention of (-)-[3H]nicotine and 125I-α-Bgt binding by the α-Bgt-coupled Sepharose was solely due to the interaction between the immobilized α-Bgt and the α-Bgt binding protein, a series of experiments were designed to elute specifically the retained α-Bgt binding protein. Triton X-100 extracts of goldfish brain, in MOPS-EDTA containing 0.1 mM phenylmethylsulfonyl fluoride and 0.02% sodium azide, were incubated with α-Bgt-coupled Sepharose overnight at 4 °C with constant rotation. The gel-extract suspension was then poured into a 5-ml disposable column and washed with 0.5 M NaCl, MOPS-EDTA, 1% Triton X-100 buffer followed by approximately 20 column volumes of MOPS-EDTA containing 1% Triton X-100 to remove any nonspecifically bound proteins. Specifically bound proteins were subsequently eluted with 5 ml each of 1 mM D-tubocurarine and 100 mM carbacholcholine (Breer et al., 1985) and dialyzed overnight against 1 mM MOPS, 1 mM EDTA, 0.1% Triton X-100, pH 7.4 at 4 °C. The dialysate was passed twice over a 1-ml DE23 ion exchange column with the binding protein being retained on the column. The column was washed twice with 5 ml of MOPS-EDTA and the binding protein recovered by elution with 1 ml of 0.5 M NaCl, MOPS-EDTA. The recovered concentrated binding protein was assayed for (-)-[3H]nicotine and 125I-α-Bgt binding as described elsewhere after control experiments established that the increased salt concentration (150 mM final) did not alter ligand binding. These experiments involved extensive manipulation of the protein and long incubation periods. The binding activity present in the preparation was monitored at various stages during the procedure to allow calculation of the losses incurred with each manipulation. As shown in Table II, a fraction of the (-)-[3H]nicotine and 125I-α-Bgt binding protein retained by the affinity gel was recoverable. Although only approximately half of the (-)-[3H]nicotine binding sites were removed from the detergent extract by α-Bgt-coupled Sepharose compared to almost all of the 125I-α-Bgt sites, approximately the same percentage of 125I-α-Bgt and (-)-[3H]nicotine binding sites which bound to the column could be recovered. The data confirm that the (-)-[3H]nicotine binding protein removed from the α-Bgt-coupled Sepharose eluent is specifically and tightly bound by immobilized α-Bgt and can be eluted specifically with cholinergic ligands. These results suggest that a portion of the (-)-[3H]nicotine binding sites may be associated with an α-Bgt binding protein.

Choline-Carboxymethyl Affinity Gel

Identical affinity chromatography experiments were attempted using choline-carboxymethyl affinity gel (Affi-Gel) rather than α-Bgt-coupled Sepharose to immobilize nAChRs from solubilized brain extract. This procedure, however, was shown to be unsatisfactory by control experiments which indicated that the eluent from the Affi-Gel column (in the absence of tissue) contained a component that was capable of inhibiting (-)-[3H]nicotine but not 125I-α-Bgt binding in Triton X-100 brain extract. This component was still present.

| Percentage of 125I-α-Bgt binding activity present | Percentage of [3H]nicotine binding activity present |
|-----------------------------------------------|-----------------------------------------------|
| Original extract                              | 100                                           |
| α-Bgt-coupled Sepharose eluent                 | 3.4 ± 2.7                                     |
| Final dialysate                                | 12.8 ± 2.5                                    |

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even following extensive washing of the gel with MOPS-EDTA buffer immediately before use. Similar control experiments with α-Bgt-coupled Sepharose eluent did not reduce either (−)-[^3H]nicotine or 125I-α-Bgt binding sites (data not shown).

**Equilibrium Binding of (−)-[^3H]Nicotine**

The binding of (−)-[^3H]nicotine to control solubilized tissue extracts and α-Bgt-coupled Sepharose eluent was investigated to determine if the two putative subtypes of (−)-[^3H]nicotine binding protein displayed different binding affinities. As previously shown (see Fig. 1), control Triton X-100 solubilized goldfish brain extract appears to possess a single class of sites which bind (−)-[^3H]nicotine with high affinity. By comparison, the results shown in Fig. 5 suggest the existence of two separable (−)-[^3H]nicotine binding sites. The data indicate that the (−)-[^3H]nicotine site which is not on the α-Bgt binding protein has a higher affinity for (−)-[^3H]nicotine with a $K_D = 3.13 \pm 0.33 \text{nM}$ (n = 5). The matched control detergent extracts from the same tissue preparations (which contained a mixture (−)-[^3H]nicotine binding sites i.e. those on the α-Bgt binding protein and those located on a protein not recognized by α-Bgt) had a $K_D = 7.39 \pm 1.02 \text{nM}$ (n = 5).

The difference in the affinity for (−)-[^3H]nicotine of the two protein subtypes was only evident following removal of one subtype with α-Bgt-coupled Sepharose because Scatchard analysis is not sufficiently sensitive to resolve this relatively minor difference in affinity in whole extract. The inability of Scatchard analysis to distinguish between two classes of sites with similar affinities has been described previously (McGonigle et al., 1988). It should be noted, however, that the affinity shift data alone are insufficient to conclude the existence of two distinct (−)-[^3H]nicotine binding protein subtypes but that these results are entirely consistent with the findings described elsewhere in this report.

**Discussion**

The data reported in this study indicate the presence of high affinity specific binding sites for (−)-[^3H]nicotine in membrane and Triton X-100-solubilized tissue preparations from whole goldfish brain. The following characteristics of the (−)-[^3H]nicotine binding fraction were determined: 1) (−)-[^3H]nicotine bound with an average $K_D$ of 4.8 and 6.4 nM to membrane fragment preparations and Triton X-100 extracts, respectively; 2) (−)-[^3H]nicotine binding was not displaceable by α-Bgt but was displaced by low concentrations of carbamoylcholine and (−)-nicotine; 3) approximately half of the (−)-[^3H]nicotine binding protein was removed from solubilized tissue extract following incubation with α-Bgt-coupled Sepharose; 4) the (−)-[^3H]nicotine binding protein which remained in the α-Bgt-coupled Sepharose eluent had a higher affinity for (−)-[^3H]nicotine than was observed in the same detergent extracts which were not incubated with α-Bgt-coupled Sepharose; and 5) similar to previously reported 125I-α-Bgt binding data, (−)-[^3H]nicotine binding sites were lost from the contralateral optic tectum following eye removal.

Sites for [^3H]Ach have previously been described in rat cortex membranes (Schwartz et al., 1982) and in chick optic lobe (Schneider et al., 1985). Reported $K_D$ values and $B_{\text{max}}$ values for [^3H]Ach in rat cortical membranes are 12.3 nM and 4.6 fmol/mg wet weight tissue (Schwartz et al., 1982) and in chick optic lobe 22.2 nM and 5.6 fmol/mg wet weight tissue (Schneider et al., 1985). In goldfish brain, [^3H]Ach binding has a $K_D$ of 13.6 nM and a $B_{\text{max}}$ of 4.1 fmol/mg wet weight tissue. [^3H]Nicotine binding sites have been demonstrated in rat and mouse brain membranes (Romano and Goldstein, 1980; Marks and Collins, 1982). These workers reported that two sites were labeled with racemic nicotine. The high affinity site was stereospecific for (−)-nicotine and seems to correspond to the [^3H]Ach binding site. More recently, Lippiello and Fernandes (1986) have confirmed that (−)-nicotine binds to a single high affinity site in rat brain which appears to be identical to the Ach binding site. (−)-[^3H]Nicotine binding in rat cortex and chick whole brain has been investigated in this laboratory and similar values to those reported here for goldfish were observed.

The α-Bgt binding site shares a number of characteristics in common with the (−)-nicotine binding site. These include an affinity for nicotinic ligands, an apparent localization of α-Bgt binding sites at synapses, and some degree of localization in CNS areas thought to be cholinergic (Arimatsu et al., 1978, 1981; Hunt and Schmidt, 1978a, 1978b; Clarke et al., 1985). In addition, considerable sequence homology exists between one of its subunits in chick brain and the α-subunit of the *Torpedo californica* electroplaque AchR (Conti-Tronconi et al., 1985). These workers conclude that the brain α-Bgt binding protein is a neuronal nAChR and is encoded by a set of genes that are different from but strongly related to those which code for the muscle nAChR.

Despite the observations cited above, serious questions remain as to the relationship between the (−)-[^3H]nicotine and 125I-α-Bgt binding sites in the CNS (i.e. do they reside on the same protein or different proteins?). These have been partially addressed in chick brain by a number of workers. Schneider et al. (1985), using a variety of chromatographic techniques, concluded that in the chick optic lobe the 125I-α-Bgt binding protein is probably a separate protein from that which is recognized by [^3H]Ach. In addition, a monoclonal antibody raised against the main immunogenic region of *Electrophorus electricus* electroplaque AChR (monoclonal antibody 35) has been used to purify a protein from chick brain which binds (−)-[^3H]nicotine but not 125I-α-Bgt (Whiting and Lindstrom, 1986). These data also suggest that the (−)-[^3H]nicotine binding fraction was not entirely identical to the [^3H]Ach binding fraction. Further work is necessary to resolve these issues.

**Fig. 5. Binding of (−)-[^3H]nicotine to control Triton X-100 brain extract and α-Bgt-coupled Sepharose eluent.** Scatchard analysis of the $K_D$ values and $B_{\text{max}}$ values (−)-[^3H]nicotine binding to control and α-Bgt-coupled Sepharose incubated detergent extracts. The extracts were incubated with gentle agitation by rotation at room temperature for 2 h with either no Sepharose (control) or α-Bgt-coupled Sepharose. 

**A** and the corresponding Scatchard plots (B). Approximately 10–15 goldfish brains were used per experiment and the affinity for (−)-[^3H]nicotine was $7.39 \pm 1.02 \text{nM}$ (n = 5) for the control extract (C) and $3.13 \pm 0.33 \text{nM}$ (n = 5) for the α-Bgt-coupled Sepharose eluent (D). The number of (−)-[^3H]nicotine binding sites ($B_{\text{max}}$) in this series of experiments was expressed as a function of total protein to compensate for any nonspecific loss on the α-Bgt-coupled Sepharose. The $B_{\text{max}}$ for control Triton X-100-solubilized tissue extract was $74.6 \pm 7.8 \text{fmol/mg protein (n = 4)}$ and $36.9 \pm 6.5 \text{fmol/mg protein (n = 4)}$ for the α-Bgt-coupled Sepharose eluent. The values given are the mean ± S.E.

4. J. M. Henley, unpublished observations.
5. J. M. Henley and R. E. Oswald, manuscript in preparation.
nicotine binding protein in chick brain, which is probably identical to the [$^{3}H$]Ach binding protein, is distinct from the 125I-α-Bgt binding protein.

Clarke et al. (1985) have used autoradiographic techniques to study the distribution of [$^{3}H$]Ach, (±)[$^{3}H$]nicotine, and 125I-α-Bgt binding sites in rat brain slices. Their data show that, in general, 125I-α-Bgt bound with a different distribution than [$^{3}H$]Ach and (±)[$^{3}H$]nicotine. There was, however, overlap of the 125I-α-Bgt and [$^{3}H$]Ach and (±)[$^{3}H$]nicotine binding sites in layer 1 of the cerebral cortex and in the superior colliculus. More recently, in solubilized rat brain, [$^{3}H$]Ach binding protein was reported not to bind appreciably to α-Bgt-coupled Sepharose (Sugiyama and Yamashita, 1986). These workers conclude that the [$^{3}H$]Ach and 125I-α-Bgt binding components in rat brain membranes appear to be distinct and separable molecules.

In higher vertebrates, electrophysiological evidence has suggested that some neurons possess α-Bgt binding sites that are not functional nAChRs. The same cells also contain nAChRs that do not bind α-Bgt but which do cause cation specific membrane channel opening following application of Ach (Patrick and Stallcup, 1977; Jacob and Berg, 1983). These data suggest that the α-Bgt binding sites may not be functional nAChRs. Conversely, there is also electrophysiological and other evidence to suggest a close relationship between the α-Bgt binding protein and functional nAChRs. This evidence includes the observations that chronic administration of nicotine causes an up-regulation of 125I-α-Bgt binding sites in rat brain (Marks et al., 1983), the effects of Ach on single units in the inferior colliculus can be blocked by α-Bgt (Farley et al., 1983), and the demonstration that some effects of light on circadian rhythms may be modulated via an α-Bgt-sensitive cholinergic pathway (Zatz and Brownstein, 1981).

In goldfish, frog, and toad brain a close connection between α-Bgt binding sites and functional nAChRs is better established. Synaptic transmission is blocked by α-Bgt (Freeman, 1977; Schmidt and Freeman, 1980; Freeman et al., 1980; Marshall, 1981), the distribution of acetylcholinesterase activity, anti-AchR antibody binding sites, and α-Bgt binding sites correspond well (Oswald et al., 1980; Sargent et al., 1984), and the α-Bgt binding protein in goldfish brain is recognized by a number of monoclonal antibodies raised against Electrophorus electroplaque nAChR (Henley et al., 1986a; 1986b). In addition, quantitatively similar numbers of α-Bgt and (−)-nicotine binding sites are lost from the contralateral optic tectum following eye removal (Henley et al., 1986b and this report).

The apparently contradictory data reported in the literature concerning the relationship of the α-Bgt binding protein to (−)-nicotine binding sites may, at least partly, be explained by a model which allows for at least two subtypes of (−)-nicotine binding sites which overlap in their 125I-α-Bgt and (−)-[$^{3}H$]nicotine binding properties. The simplest interpretation of the data presented in this report is that two related proteins may be present, one which binds only (−)-[$^{3}H$]nicotine and a second that binds both (−)-[$^{3}H$]nicotine and 125I-α-Bgt. A third subtype which binds only 125I-α-Bgt may also exist as suggested by Clarke et al. (1985). If these binding sites represent neuronal nAChRs, this hypothesis is supported by the observation that at least two different mRNA species which have homologies with muscle and PC12 nAChR are present in rat brain (Goldman et al., 1986). An alternative possibility may be that membrane-bound and intracellular precursor (−)-nicotine binding proteins possess different sensitivity to α-Bgt and the two “subtypes” observed represent these two populations. On solubilization, both the precursor and mature forms of the (−)-nicotine binding protein are measured thus giving rise to the two classes of (−)-nicotine binding sites.

The affinity gel experiments indicate that although there is some (−)-[$^{3}H$]nicotine binding activity associated with the protein immobilized on the α-Bgt-coupled Sepharose, not all the (−)-[$^{3}H$]nicotine binding protein was retained. Approximately 50% of the (−)-[$^{3}H$]nicotine binding protein was retained on α-Bgt-affinity Sepharose, suggesting that a considerable portion of the α-Bgt binding protein also binds (−)-[$^{3}H$]nicotine. Although a sizable subpopulation of (−)-[$^{3}H$]nicotine binding protein also binds 125I-α-Bgt, it remains unclear if all or only a fraction of the α-Bgt binding protein binds (−)-[$^{3}H$]nicotine. This partial co-localization model is supported by the finding that 125I-α-Bgt binding is almost totally removed by preincubation with α-Bgt-coupled Sepharose but about half of the (−)-[$^{3}H$]nicotine binding protein remains. Furthermore, the different binding affinities for (−)-[$^{3}H$]nicotine demonstrated in this report support the subtype hypothesis. Following incubation with α-Bgt-coupled Sepharose the affinity of the nicotine binding protein remaining in the eluent for (−)-[$^{3}H$]nicotine was increased compared to controls (see Fig. 5).

In summary, the data presented in this report strongly support the hypothesis that at least two separable (−)-nicotine binding proteins are present in goldfish brain. As described above, one of these subtypes possesses both a (−)-[$^{3}H$]nicotine and 125I-α-Bgt site(s) whereas the other binds only (−)-[$^{3}H$]nicotine. An attractive possibility is that these binding proteins represent subtypes of neuronal nicotinic acetylcholine receptors.

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REFERENCES

Arimatsu, Y., Seto, A., and Amano, T. (1978) Brain Res. 147, 165-169

Bradford, M. M. (1976) Anal. Biochem. 72, 248-254

Breer, H., Kleene, R., and Hinz, G. (1983) J. Neurosci. 5, 3386-3392

Brown, D. A., and Fumagalli, L. (1977) Brain Res. 129, 165-168

Bruns, R. F., Lawson-Wendling, K., and Pugsley, T. A. (1983) Anal. Biochem. 132, 74-81

Carbonetto, S. T., Fischbough, D. M., and Muller, K. J. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 105-110

Cheng, Y., and Prusoff, W. H. (1973) Biochem. Pharmacol. 22, 3099-3108

Clarke, P. B. S., Schwartz, R. D., Paul, S. M., Pert, C. B., and Pert, A. (1985) J. Neurosci. 5, 1307-1315

Coni-Tronconi, B. M., Dune, S. M. J., Barnard, E. A., Dolly, J. O., Lai, F. A., Ray, N., and Raferty, M. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5208-5212

Costa, L. G., and Murphy, S. D. (1983) J. Pharmacol. Exp. Ther. 226, 392-397

Farley, G. E., Orley, B. J., Javel, E., and Gorga, M. P. (1983) Hear. Res. 11, 73-91

Freeman, J. A. (1977) Nature 269, 218-222

Freeman, J. A., Schmidt, J. T., and Oswald, R. E. (1980) Neuroscience 5, 929-942

Goldman, D., Simmons, D., Swanson, L. W., Patrick, J., and Heinemann, S. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4076-4080

Hanke, W., and Breer, H. (1986) Nature 321, 171-174

Henley, J. M., Mynlieff, M., Lindstrom, J. M., and Oswald, R. E. (1986a) Brain Res 364, 404-408

Hug, J. M., Lindstrom, J. M., and Oswald, R. E. (1986b) Science 232, 1627-1629

Horne, W. A., Weiland, G. A., and Oswald, R. E. (1986) J. Biol. Chem. 261, 3588-3594

Hunter, S. P., and Schmidt, J. (1978a) Brain Res. 142, 152-159

Hunter, S., and Schmidt, J. (1978b) Brain Res. 157, 219-222

Jacob, M. H., and Berg, D. R. (1983) J. Neurosci. 3, 289-271

Karlin, A. (1980) in The Cell Surface and Neuronal Function (Cotman, C. W., Poste, G., and Nicolson, G. L., eds) pp. 191-260, Elsevier,
Amsterdam
Kemp, G., Bentley, L., McNamee, M. G., and Morley, B. J. (1985) *Brain Res.* **347**, 274–283
Lippiello, P. M., and Fernandes, K. G. (1986) *Mol. Pharmacol.* **29**, 448–454
Marks, M. J., and Collins, A. C. (1982) *Mol. Pharmacol.* **22**, 554–564
Marks, M. J., Burch, J. B., and Collins, A. C. (1983) *J. Pharmacol. Exp. Ther.* **226**, 817–825
Marshall, L. M. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 1948–1952
Martino, A. M., Hamui, V., and Kellar, K. J. (1985) *Soc. Neurosci. Abstr.* **11**, 93
McGonigle, P., Neve, K. A., and Molinoff, P. B. (1986) *Mol. Pharmacol.* **30**, 329–337
Oswald, R. E., and Freeman, J. A. (1979) *J. Biol. Chem.* **254**, 3419–3426
Oswald, R. E., Schmidt, J. T., Norden, J. J., and Freeman, J. A. (1980) *Brain Res.* **187**, 113–127
Oswald, R. E., and Freeman, J. A. (1981) *Neuroscience* **6**, 1–14
Patrick, J., and Stallcup, W. B. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 4689–4692
Ravdin, P. M., and Berg, D. K. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 2072–2076
Reynolds, J. A., and Karlin, A. (1978) *Biochemistry* **17**, 2035–2038
Romano, C., and Goldstein, A. (1980) *Science* **210**, 647–649
Sargent, P. B., Pike, S. H., Tsavalier, L., and Lindstrom, J. M. (1984) *Soc. Neurosci. Abstr.* **10**, 935
Schechter, N., Francis, A., Deutsch, D. G., and Gazzaniga, M. S. (1979) *Brain Res.* **166**, 57–64
Schneider, M., Adee, C., Betz, H., and Schmidt, J. (1985) *J. Biol. Chem.* **260**, 14506–14512
Schwartz, R. D., McGee, R., and Kellar, K. J. (1982) *Mol. Pharmacol.* **22**, 56–62
Schwartz, R. D., and Kellar, K. J. (1983) *Science* **220**, 214–216
Schmidt, J. T. (1979) *Proc. R. Soc. Lond. B Biol. Sci.* **205**, 287–306
Schmidt, J. T., and Freeman, J. A. (1980) *Brain Res.* **187**, 129–142
Sugiyama, H., and Yamashita, Y. (1986) *Brain Res.* **373**, 22–26
Tumosa, N., and Stell, W. K. (1986) *J. Comp. Neurol.* **244**, 267–275
Whiting, P. J., and Lindstrom, J. M. (1986) *Biochemistry* **25**, 2092–2093
Zatz, M., and Brownstein, M. J. (1981) *Brain Res.* **213**, 438–442