The Journal of Neuroscience, February 1990, *10*(2): 670-683

Multiple Nicotinic Acetylcholine Receptor Genes Are Expressed in Goldfish Retina and Tectum

Keith Cauley, Bernard W. Agranoff, and Daniel Goldman

Mental Health Research Institute and Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109

cDNAs encoding a novel nAChR structural subunit (GFnα-3) and a ligand-binding subunit (GFα-3) have been isolated from a goldfish retina cDNA library. The protein encoded by GFnα-3 shares 88% amino acid similarity with that encoded by GFnα-2, a structural subunit gene previously identified to be expressed in this system (Cauley et al., 1989). The ligand-binding subunit (GFα-3) is likely the goldfish homolog of the rat α-3 gene (Boulter et al., 1986). Northern blots and S1 protection experiments show that GFnα-3 and GFα-3 genes are expressed in retina and brain. GFnα-3 identifies multiple RNAs differing in their 3′ untranslated regions. *In situ* hybridization analysis demonstrates GFnα-3, GFnα-2, and GFα-3 expression by cells of the retinal ganglion cell layer. Unlike GFnα-2 and GFα-3, GFnα-3 is expressed at highest levels by cells of the retina's inner nuclear layer. In the optic tectum, both GFα-3 and GFnα-3 genes are expressed by cells of the periventricular zone, as well as more superficial layers. These results suggest the presence of multiple nAChR systems in retina and tectum. In addition, they indicate that tectal nAChRs may arise from remote (ganglion cell) as well as local (tectal cell) synthesis.

The retina is one of the most experimentally accessible regions of the brain for biological studies. We have selected the retina as a model system to study the influence of cell–cell interactions on the expression of neural nicotinic acetylcholine receptors (nAChRs). ACh and nAChRs are likely to be involved in synaptic communication within the retina and possibly between retina and brain. ACh has long been implicated as a neurotransmitter in the vertebrate retina (Ames and Pollen, 1968; see reviews by Graham, 1974, and Neal, 1976). Identification of acetylcholinesterase and choline acetyltransferase activities evidence the presence of cholinergic synapses in the retinas of several species (Francis, 1953; Ross and McDougal, 1976). These markers, together with ^{3}H-choline uptake studies, identify a subpopulation of retinal amacrine cells that are cholinergic (Masland and Mills, 1979). Pharmacological and electrophysiological studies indicate the existence of nicotinic cholinergic synapses at amacrine-ganglion cell contacts (Masland and Ames, 1976; Negishi et al., 1978; Ariel and Daw, 1982). Patch-clamp recording confirms that at least some ganglion cells express functional receptors responsive to classical nicotinic agonists and antagonists (Lipton et al., 1987). Monoclonal antibody studies and cDNA cloning show the expression of nicotinic receptors by cells of the retinal ganglion cell layer (Keyser et al., 1988; Cauley et al., 1989; Sargent et al., 1989). Ganglion cells that have complex receptive fields receive a light-driven cholinergic input (Masland and Livingstone, 1976; Masland et al., 1984). This input seems to modulate the activity of some physiological classes of ganglion cells (Masland and Ames, 1976; Negishi et al., 1978; Ariel and Daw, 1982; Glickman et al., 1982). In order to determine the role nAChRs play in the retina we have begun isolating and characterizing retinal nAChR cDNA clones (Cauley et al., 1989).

Alpha-bungarotoxin (α-BTX) has traditionally been used to identify nAChRs in vertebrate skeletal muscle (Changeux, 1980). However, in the CNS this toxin has been shown to bind sometimes to sites distinct from those that bind nicotine (Clark et al., 1985) or certain nAChR antibodies (Patrick and Stallcup, 1977). Goldfish brain possesses at least 2 classes of nicotine binding sites, one of which also binds α-BTX (Henley and Oswald, 1987). One of these α-BTX binding molecules is synthesized by retinal ganglion cells and transported to the optic tectum (Henley et al., 1986a). In goldfish retina α-BTX binding localizes to the inner and outer plexiform layers, and this binding can be competed by nicotine (Yazulla and Schmidt, 1976; Schwartz and Bok, 1979). The function of these toxin binding molecules and their relationship to each other and to the other nicotinic receptor types are not known.

In order to determine the number and function of different nAChRs expressed in the visual system it is necessary to isolate and characterize these molecules. This is most easily accomplished by cDNA cloning. Using this strategy we have previously identified a novel nAChR structural subunit, GFnα-2 (goldfish non-alpha 2), expressed at high levels by retinal ganglion cells (Cauley et al., 1989). Here we report the cloning and characterization of 2 more members of this gene family—GFα-3, a putative goldfish homolog of the rat α-3 gene (Boulter et al., 1986), and GFnα-3, a gene encoding a new structural subunit with unexpectedly high homology with the GFnα-2 protein.

Materials and Methods

Isolation of cDNA clones. Common goldfish (*Carassius auratus*) were used in these studies. A λgt10 cDNA library was prepared from goldfish retinal RNA and screened with a mixed α-subunit probe as previously described (Cauley et al., 1989). This probe consisted of nick-translated

Received July 18, 1989; revised Aug. 31, 1989; accepted Sept. 6, 1989.

We thank John Bouchey for help with DNA sequencing and Dr. P. Hitchcock for help with propidium iodide retrograde labeling of retinal ganglion cells, and Dr. R. Davis for advice on goldfish neuroanatomy. We also thank Drs. S. Heinemann and J. Patrick for the rat neural α-4 cDNA clone, and Dr. N. Davidson for the *Torpedo* α-subunit cDNA.

This work was supported by grants from the National Institutes of Health and the Lucille P. Markey Charitable Trust to B. W. Agranoff and D. Goldman.

Correspondence should be addressed to Daniel Goldman, Mental Health Research Institute, 205 Washtenaw Place, Ann Arbor, MI 48109.

Copyright © 1990 Society for Neuroscience 0270-6474/90/100670-14$02.00/0

(Rigby et al., 1977) cDNAs encoding the α-subunits of the *Torpedo* electric organ nAChR (Noda et al., 1982), kindly provided by Dr. Norman Davidson (California Institute of Technology), the rat muscle nAChR, and the rat neural nAChR α-4 subunit (Goldman et al., 1987). This screening resulted in the isolation of 6 cDNAs that encode 3 different goldfish neural nAChR subunits. One of these receptor subunit cDNA clones, GFnα-2, has been characterized (Cauley et al., 1989).

Northern blot analysis and S1 nuclease protection experiments. Northern blots and S1 nuclease protection experiments were carried out as previously described. Briefly, RNA was isolated using a modification of the guanidinium isothiocyanate procedure (Chirgwin et al., 1979). Poly(A)+ RNA was selected over an oligo (dT) cellulose column (Aviv and Leder, 1972). For Northern blots 2.5 μg of retinal and 5 μg of brain poly(A)+ RNA and 10 μg of poly(A)− RNA from each tissue were electrophoresed on denaturing agarose gels, then transferred to a Gene Screen Plus membrane. Membranes were hybridized under low-stringency conditions (~40°C below the T_m) with nick-translated cDNA probes. Following hybridization, blots were washed at a stringency of ~40°C below the T_m and exposed to x-ray film with an intensifying screen at −80°C for 6–12 hr.

S1 nuclease digestions of heteroduplexes formed between poly (A)+ RNA and M13 subclones of GFnα-3 and the GFα-3 cDNA were also carried out as previously described (Goldman et al., 1985). Retinal poly (A)+ RNA (5 μg) was hybridized with M13 subclones containing complementary DNA corresponding to either the full-length cDNA or to subclones containing deletions at their 5′ end (see Fig. 5A). Deletions were generated using T4 polymerase (Dale et al., 1985). These latter clones contained GFnα-3 3′ DNA extending 5′ for 2498, 1918, 1658, or 950 bases. Following hybridization of these clones to the RNA, S1 nuclease was added and those hybrids surviving S1 digestion were size-fractionated by electrophoresis through a denaturing 1.2% agarose gel. Protected nucleic acids were transferred to a Gene Screen Plus membrane, hybridized with a nick-translated GFnα-3 or GFα-3 probe, and washed at ~40°C below the T_m. Blots were exposed to x-ray film overnight at −80°C with an intensifying screen. Nick-translated probes were radiolabeled to a specific activity of ~10^8 cpm/μg. The GFnα-3 Northern blot and GFα-3 S1 nuclease protection blot autoradiograms were quantitated by densitometry using a Loats Associates Inc. image-analysis system.

Fluorescent back-labeling of retinal ganglion cells. Retinal ganglion cells were identified by fluorescence following retrograde labeling via the optic nerve using propidium iodide as a fluorescent tracer (Woolf et al., 1983; Tumosa and Stell, 1986). Propidium iodide (Sigma) was made up to 3% in 1% DMSO. Fish were anesthetized as previously described (Heacock and Agranoff, 1982), optic nerves were transected, and a small piece of propidium iodide-impregnated Gel-Foam was placed upon the proximal stump of the optic nerve. The eye was carefully repositioned and sealed with Super Glue. Three days later, retinas were removed and fixed as for *in situ* hybridization. Retinas were sectioned at 15 μm, and back-labeled cells visualized using a Zeiss Axiophot microscope equipped with rhodamine optics.

In situ *hybridization. In situ* hybridization was performed as previously described (Cauley et al., 1989). In brief, goldfish retinas and brains were removed and fixed in ice-cold 4% paraformaldehyde in PBS (145 mM NaCl, 10 mM phosphate buffer, pH 7.4) for 2 hr. Tissues were then incubated in 20% sucrose/PBS at 4°C overnight. Sections, 15 μm thick, were cut and mounted on gelatin-chromalum subbed, polylysine-coated slides. Prior to *in situ* hybridization, sections were digested with proteinase K (5 μg/ml, 37°C, 5 min) and acetylated with acetic anhydride (0.25% in triethanolamine HCl, pH 8.0, 10 min, room temperature). Sections were hybridized with single-stranded ^{35}S-labeled RNA probes, prepared by run-off transcription of linearized recombinant plasmids. Hybridization solution was brought to 5 × 10^4 cpm/μl with the ^{35}S-UTP-labeled RNA probes. The GFα-3 probe was made from a Pst 1 linearized pGEM-4 vector containing the 909 bp GFα-3 cDNA inserted into the EcoR 1 site. The GFnα-2 probe was made from a pGEM-4 vector harboring an EcoR 1/Dra 1 1650 bp subclone of GFnα-2 (Cauley et al., 1989). This subclone contains the GFnα-2 cDNA lacking its poly(A) tail and some 3′ untranslated sequence. The GFnα-3 probe was made from an EcoR 1 linearized pGEM-4 vector containing a 1594 bp Bgl II fragment subcloned into the BamH 1 site of pGEM-4. This fragment extends from nucleotide 107 to 1601 of the GFnα-3 cDNA. Antisense probes for GFnα-2 and GFnα-3 were transcribed with T7 RNA polymerase and sense orientation probes with SP6 RNA polymerase (Melton et al., 1984). The GFα-3 antisense probes were transcribed with SP6 RNA polymerase. Posthybridization treatments included a wash in 50% formamide, 2× SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.0) at 55°C (Fontaine et al., 1988) and an RNase digestion to reduce background (Cox et al., 1984). Controls for nonspecific hybridization in *in situ* hybridization experiments included the use of sense-strand probes and pretreating sections with RNase before hybridization with antisense probes. Slides were then dehydrated, dipped in Nuclear Track Emulsion NTB2, air-dried, and exposed at 4°C. Retinal sections were exposed 1–2 weeks, and brain sections were exposed 2–4 weeks. After developing, sections were stained with hematoxylin and eosin B. Low-magnification coronal brain sections were visualized using a Wild Makroskop M420 equipped with darkfield optics, and higher magnifications were visualized with a Zeiss Axiophot.

DNA sequence determination and comparison. DNA sequencing was performed using the dideoxynucleotide chain termination method (Sanger et al., 1977). cDNAs were subcloned into M13 bacteriophage vectors mp18 and mp19. Unidirectional deletions were generated with T4 DNA polymerase (Dale et al., 1985). Both strands of DNA were completely sequenced. Sequence comparisons were made with the University of Wisconsin Genetics Computer Group sequence analysis software package version 6.0. The GAP program using the algorithm of Needleman and Wunsch (1970) was used with "default" parameter settings.

Results

Isolation of goldfish neural nAChR cDNAs

The vertebrate retina expresses both nicotine and α-BTX binding molecules (Masland and Ames, 1976; Vogel and Nirenberg, 1976; Betz and Müller, 1981). In goldfish, members of these classes of molecules are found in the inner and outer plexiform layers of the retina (Yazulla and Schmidt, 1976; Schwartz and Bok, 1979), along the optic nerve (Schwartz et al., 1980), and throughout regions of the optic tectum where retinal afferents synapse (Henley et al., 1986b). We are using cDNA cloning to identify these molecules (Cauley et al., 1989).

A goldfish retinal cDNA library was screened with a radiolabeled mixed receptor probe (see Materials and Methods). Screening 5 × 10^5 recombinants resulted in the purification of 6 cDNAs representing 3 classes of molecules. One class representing a new nAChR subunit, GFnα-2, has been characterized (Cauley et al., 1989). The other 2 classes are represented by clones GFnα-3 (goldfish non-alpha 3) and by GFα-3 (goldfish alpha 3), and are characterized in this study.

GFnα-3

Clone GFnα-3 is 2698 nucleotides in length, with an open reading frame of 1398 bp (Fig. 1). This open reading frame begins with an initiator ATG at position −84 and ends with a stop codon, TAA, at position 1315. The 5′ and 3′ ends of the clone contain 144 and 1156 bases of untranslated sequence, respectively. There are 4 short open reading frames in the 5′ untranslated sequence, with initiator ATGs at positions −193, −158, −131, and −113, which may influence the translatability of this transcript (Kozak, 1989). Based on comparison of the deduced amino acid sequence of GFNα-3 with other nAChR subunit sequences (Fig. 2), and hydrophobicity analysis (Kyte and Doolittle, 1982), and taking into account the sequence patterns around the signal cleavage sight (Perlman and Halvorson, 1983; von Heijne, 1983), we have designated the amino terminus of the mature GFnα-3 protein to be an alanine residue. This results in the identification of the first 28 residues as comprising a leader peptide. The mature protein is 438 amino acids and has a predicted molecular weight of 53,783. Four potential poly (A) addition signal sequences are found within the 3′ untranslated sequence (underlined in Fig. 1; Proudfoot and Brownlee, 1976; Birnstiel et al., 1985).

Comparison of the deduced amino acid sequence of clone

```
-228  TGA TCG TTT TCC AAA ACG GCC CGG TTT CCA GCC ATA TGA ATA AAA AAA CAA TAT ACC CCG  -169

-168  TTT TTT GTC GAT GAC AGG GAC TCG CTA TTC AAC CAG CAT GAA ATA ATT CTA TTG CAT GAA  -109

                                          Met Lys Leu Gln Ile Ser Gly Leu Leu Leu Val Thr
-108  GGA ATT GGA GCC GGG AAA GAC AGC ATG AAG TTA CAA ATC TCC GGT CTT TTG CTC GTA ACT  -49
                                                                       1
      Ala Val Ala Tyr Ala Thr Ile Glu Ala Pro Glu Glu Phe Val Ser Leu Ala Glu Met Glu
 -48  GCA GTA GCC TAT GCA ACG ATA GAA GCT CCT GAG GAG TTT GTC TCA CTG GCT GAG ATG GAG  12

      Asp Thr Leu Leu Arg Asn Leu Phe Arg Gly Tyr Gln Lys Trp Val Arg Pro Ile Leu His
  13  GAC ACG TTG TTG AGG AAT CTG TTT CGG GGC TAT CAG AAG TGG GTG AGA CCT ATC CTT CAC  72

      Ala Asn Asp Thr Ile Thr Val Arg Phe Gly Leu Lys Ile Ser Gln Leu Val Asp Val Asp
  73  GCC AAC GAC ACG ATT ACC GTT CGC TTT GGT CTG AAG ATC TCG CAG CTG GTG GAT GTG GAT  132

      Glu Lys Asn His Leu Met Thr Thr Asn Val Trp Leu Trp Gln Glu Trp Thr Asp Tyr Lys
 133  GAA AAG AAT CAC CTA ATG ACG ACA AAT GTC TGG TTG TGG CAG GAG TGG ACA GAC TAT AAG  192

      Leu Arg Trp Asn Pro Glu Asp Tyr Gly Gly Ile Thr Ser Ile Arg Val Pro Ser Glu Thr
 193  CTT CGG TGG AAT CCG GAA GAT TAC GGT GGA ATC ACA TCC ATC AGA GTG CCC TCA GAA ACC  252

      Ile Trp Leu Pro Asp Ile Val Leu Tyr Glu Asn Ala Asp Gly Arg Phe Glu Gly Ser Leu
 253  ATC TGG CTA CCA GAC ATT GTC CTT TAC GAG AAT GCT GAT GGC CGA TTT GAG GGC TCT CTG  312

      Met Thr Lys Ala Ile Val Arg Phe Asn Gly Thr Ile Met Trp Thr Pro Pro Ala Ser Tyr
 313  ATG ACC AAA GCC ATT GTA AGG TTT AAT GGT ACC ATC ATG TGG ACT CCT CCT GCC AGC TAT  372

      Lys Ser Ser Cys Thr Met Asp Val Thr Phe Phe Pro Phe Asp Arg Gln Asn Cys Ser Met
 373  AAA TCC TCC TGT ACC ATG GAT GTC ACC TTT TTC CCC TTC GAC AGG CAG AAC TGC TCG ATG  432

      Lys Phe Gly Ser Trp Thr Tyr Asp Gly Thr Met Val Asp Leu Thr Leu Leu Asp Ala Tyr
 433  AAG TTT GGA TCG TGG ACG TAC GAT GGC ACT ATG GTG GAC CTG ACT CTG TTA GAC GCA TAT  492

      Val Asp Arg Lys Asp Phe Phe Asp Asn Gly Glu Trp Glu Ile Leu Asn Ala Thr Gly Gln
 493  GTA GAC CGT AAA GAC TTC TTT GAC AAT GGC GAA TGG GAA ATA CTC AAC GCC ACC GGC CAG  552

      Arg Gly Ser Arg Arg Asp Gly Ile Tyr Ser Tyr Pro Tyr Val Thr Tyr Ser Phe Ile Leu
 553  AGG GGA AGT CGG CGT GAC GGC ATT TAC TCT TAT CCT TAC GTC ACA TAT TCG TTC ATT CTG  612

      Lys Arg Leu Pro Leu Phe Tyr Thr Leu Phe Leu Ile Ile Pro Cys Leu Gly Leu Ser Phe
 613  AAA CGT CTT CCT CTG TTT TAC ACA CTC TTT CTC ATC ATC CCA TGT CTG GGT CTG TCG TTT  672

      Leu Thr Val Leu Val Phe Tyr Leu Pro Ser Asp Glu Gly Glu Lys Leu Leu Leu Ser Thr
 673  CTC ACG GTT CTG GTC TTC TAC CTC CCC TCG GAT GAA GGA GAA AAG CTG CTC CTC TCC ACC  732

      Ser Val Leu Val Ser Leu Thr Val Phe Leu Leu Val Ile Glu Glu Ile Ile Pro Ser Ser
 733  TCC GTC CTT GTG TCT CTC ACT GTG TTT CTT CTT GTC ATC GAA GAG ATC ATT CCA TCT TCC  792

      Ser Lys Val Ile Pro Leu Ile Gly Glu Tyr Leu Leu Phe Ile Met Ile Phe Val Thr Phe
 793  TCT AAA GTC ATT CCT TTG ATT GGA GAG TAT CTG CTT TTT ATC ATG ATC TTC GTC ACA TTC  852

      Ser Ile Ile Val Thr Leu Phe Val Ile Asn Val His His Arg Ser Ser Ala Thr Tyr His
 853  TCG ATC ATC GTG ACT TTG TTC GTC ATC AAC GTC CAT CAT CGT TCT TCT GCC ACG TAC CAT  912

      Pro Met Ala Pro Trp Val Lys Ser Leu Phe Leu Gln Arg Leu Pro Arg Leu Leu Cys Met
 913  CCA ATG GCG CCG TGG GTT AAG AGT CTT TTC CTT CAG AGA TTA CCC AGA CTG CTC TGC ATG  972

      Arg Gly His Thr Asp Arg Tyr Gln Tyr Pro Asp Ile Glu Leu Arg Ser Pro Glu Leu Lys
 973  AGG GGA CAT ACT GAT CGC TAT CAG TAC CCA GAC ATC GAG CTG CGG AGC CCA GAG CTA AAA  1032

      Arg Gly Met Lys Lys Gly Gln Gln Lys Ser Ala Gly Gly Gly Arg Gly Gly Leu Lys Glu
1033  CGA GGG ATG AAG AAA GGG CAA CAG AAG AGT GCC GGA GGA GGG CGA GGA GGG TTA AAG GAA  1092

      Asp Glu Asn Gln Ala Trp Ile Ala Leu Leu Glu Lys Ala Thr His Ser Val His Tyr Ile
1093  GAT GAG AAT CAG GCC TGG ATT GCT CTG TTG GAG AAA GCC ACT CAC TCA GTG CAC TAC ATT  1152

      Ser Arg His Ile Lys Lys Glu His Phe Ile Arg Glu Val Val Gln Asp Trp Lys Phe Val
1153  TCC AGA CAC ATC AAG AAG GAA CAC TTC ATC AGA GAG GTT GTT CAG GAT TGG AAG TTT GTG  1212

      Ala Gln Val Leu Asp Arg Ile Phe Leu Trp Val Phe Leu Thr Ala Ser Val Leu Gly Thr
1213  GCT CAG GTA TTG GAC CGG ATC TTC CTC TGG GTC TTT CTT ACA GCT TCT GTG CTT GGG ACC  1272

      Ile Leu Ile Phe Thr Pro Ala Leu His Met Tyr Leu Ser Thr End
1273  ATC CTC ATC TTC ACC CCA GCT CTG CAC ATG TAC CTC AGC ACA TAA AAA AAA ACA GCC TAC  1332

1333  CAA ATC TCC TTT ACC CTC GAC AAC ATT CTT ATG ACC CCT TTG AAA TAT ATA GTT CCC ACA  1392
1393  ATA TCA CAG GAA TCC TTC TAA ACG TAT TTC TGA CCT AGC ATA TCT GTA ATC CTC CTA AAA  1452
1453  TTC CAG TCT CCT AGC TCT CCT TTC AGC TGT GAA AAA AAG TCA AAC TTT CAA AAC ATT TTC  1512
1513  AAC CCG TTT AAC ACA TAA CTA AGA ATC ATC TTA TGA AGT TGG GGT TGT TTG GAT TTG TGT  1572
1573  AAT CTC TCC AAA TGG GTC ATG GGT TTA AAG ATC TTC AAT AAT TTG ACT TTA ATT ATC ACT  1632
1633  ACT GTG ATG TAA AAG GTG AAC CAC ATC TTT CAT GCC TCT TAT TTC ACT TGG TAT CAT TCA  1692
1693  TTC CTG TAT TTA TGT ATT TAT TTA TTT AGG GTG GGT AAA AAT TGT GTC AGT GTT CAT TTA  1752
1753  AGT AAT ATT TTA CAG TAA TAT TTT ATC ATT CCG GTC ATT AGG AAA CAA ACC TAT TCA ATA  1812
1813  GAT TAA AAT TCC ATC TGT ATG CAA TAA ATA TTA ATT TTT TAC ACT ATA CAG ATA TTA CTT  1872
1873  CTG GAT GAT GAT AGC ATG GAG AAG ATT AGT AGA TTG GAT AGT AAT TAT ATG AAA TTA TAA  1932
1933  CAG TAT CTC TAT TAT TGG GAA AGG TTC CTT ACT GTA CAG TCT TGC ATA TAT TGT CCT AGT  1992
1993  TTT TAG TAC TGT TAA ATG CAT GTA GTA TCA TGC CTT TGG ATG ACA GTA TGC ATA TGT AAA  2052
2053  TGT TAT GCA GAT GAG GCC ATG CAT AAT ACA AAG TGC ATG TGT ATG TAT CTA CAA TCT TCC  2112
2113  ACA ATC TGG GAT TTA TAA AAA AAG ACA TTT ACA CAG ATG CAT ATA CTG TAT GGT GCA TAT  2172
2173  ATG GAT TTA TAA ATC TCA ATA TCT CCC ATT TGT GCT TAT AGG ATG AAG TCT ACA CCA CAT  2232
2233  GTT AAA CAT TCT TAT CCC TAA ATC CTT GGA GAT ATT GTC GCT GAT TCA AGT GGG AGA GAT  2292
2293  TAC TTG ATC ACA GCT TGT GTT TGG CAT CAT AAG CAA GGA CTT TAT TAT GAT ATC CAT CTT  2352
2353  TAT TTC TGT TCA TTC ACA CTG GTC AGT AAA TGG AAA TGT CAT TGT ATC AGT TTT TTG TTG  2412
2413  TTT GAA TGT CTA ACA TGT GTT TCA TGT AAA TAA CCT TGT AAG TGA TCT AAA AAA AAA A..3' 2470
```

Figure 1. Nucleotide and deduced amino acid sequence of cDNA clone GFnα-3. Nucleotides are numbered in the 5′ to 3′ direction. The amino acid designated 1 (Ala) represents the putative amino-terminal residue in the mature protein. Sequences extending 5′ to base 1 are designated with negative numbers and include residues encoding the putative signal peptide and 5′ untranslated sequence. Polyadenylation signal sequences are underlined in the 3′ untranslated region.

GFnα-3 with the muscle and neural nAChR sequences shows that this clone encodes a protein that is a member of the nAChR gene family (Fig. 2, Table 1). GFnα-3 is remarkably similar to GFnα-2, with an overall amino acid sequence similarity of 88% (Table 1). These 2 proteins are more similar to the rat β3 protein (Deneris et al., 1989; 85% for GFnα-3, 82% for GFnα-2) than to any other nAChR subunit identified to date (Table 1). In fact, no other known pair of nAChR subunits show this level of similarity.

Analysis of the GFnα-3 protein sequence indicates that it contains many structural features common to all neural nAChR subunits sequenced to date (Barnard et al., 1987). These include (1) four hydrophobic putative transmembrane domains; (2) an extracellular β-loop structure occurring before the first hydrophobic domain, formed by cysteines 128 and 142, with a turn induced by the conserved proline at position 136; (3) 2 putative cytoplasmic N-linked glycosylation sites at positions 26 and 141; and (4) a hydrophilic domain situated between the third and fourth hydrophobic domains. In addition to those putative glycosylation sites mentioned above, the GFnα-3 protein also has N-linked glycosylation consensus sites at amino acid positions 113 and 180.

The protein encoded by GFnα-3 lacks adjacent cysteines corresponding to positions 191 and 192 of the *Torpedo* nAChR α-subunit (Noda et al., 1982). These cysteines are found in all nAChR α-subunits sequenced to date and are believed to be close to the agonist binding domain (Kao and Karlin, 1986). In this respect, GFnα-3 is more similar to the structural non-α (nα) subunits of muscle and neural nAChRs. Based on the absence of these adjacent cysteine residues in GFnα-3, and the conservation of the many structural domains between this protein and all other neural nAChR subunits, we propose that GFnα-3 represents a structural subunit of a novel class of neural nAChRs.

GFα-3

Since 2 novel nAChR subunit genes have been identified in goldfish (GFnα-2, Cauley et al., 1989, and GFnα-3, this paper), it was important to determine whether other previously identified members of the vertebrate nAChR gene family are also expressed in fish. The identification of these genes would imply that a similar gene family is expressed in fish and higher vertebrates. Clone GFα-3 represents one such gene. GFα-3 is a partial cDNA whose deduced amino acid sequence indicates that it may represent the goldfish homolog of the rat α-3 gene (Boulter et al., 1986; Fig. 3).

GFα-3 is 909 bp in length containing an open reading frame coding for a 303 amino acid polypeptide (Fig. 3). GFα-3 represents the 5' end of the gene coding for amino acids −21 through 281, based on the rat α-3 sequence (Boulter et al., 1986). This region includes the 2 cysteine residues at positions 192 and 193 that are presumed to be close to the ligand-binding domain. GFα-3 possesses cysteines 128 and 142, common to all known nAChR subunits. The putative glycosylation sites at amino acid positions 23 and 141, common to the neural nAChR subunits, are also present. Comparison of the deduced GFα-3 amino acid sequence with known nAChR subunits shows GFα-3 to be most similar to the neural α-3 subunit [89% similarity with rat α-3 (Fig. 3) and 93% similarity with chicken α-3 (Nef et al., 1988)]. This degree of homology is higher than that of any of the rat subunits with each other and is most consistent with GFα-3 representing the goldfish homolog of the rat and chicken α-3 gene.

Table 1. Percentage of amino acid similarity between non-alpha nAChR subunits

Subunit	GFnα-3	GFnα-2	Ratβ-3	Ratβ-2	Ratβ-4	ARDnα
GFnα-3	100					
GFnα-2	88	100				
Ratβ-3	85	82	100			
Ratβ-2	62	64	64	100		
Ratβ-4	57	56	59	67	100	
ARDnα	63	61	63	61	55	100

Similarity was determined using University of Wisconsin Genetics Computer Group Gap program. Amino acid sequences are GFnα-3 (this report), GFnα-2 (Cauley et al., 1989), Ratβ-3 (Deneris et al., 1989), Ratβ-2 (Deneris et al., 1988), Ratβ-4 (clone SCG 3, Isenberg and Meyer, 1989), and ARDnα (Hermans-Borgmeyer et al., 1986).

nAChR gene expression in goldfish retina and brain

GFnα-3 identifies multiple RNAs expressed in retina and brain

The level of expression of RNAs homologous to the GFnα-3 cDNA was assayed by Northern blot (Fig. 4). This experiment showed GFnα-3 to hybridize to several different RNAs in retina and brain, with the majority of hybridization at 2.7 kb in both tissues. Densitometric integration of the autoradiogram showed that retina has about a 12-fold higher level of GFnα-3 expression per microgram of RNA than brain.

S1 nuclease protection experiments were used to determine if any of the RNAs seen hybridizing with the GFnα-3 probe on Northern blots contained regions that were identical or very similar to the GFnα-3 cDNA. This experiment resulted in the protection of 4 different retinal RNAs of approximately 2.7, 1.9, 1.7, and 0.8 kb (Fig. 5*B*, lane 2698+). An identical pattern was seen when this experiment was performed using brain RNA, although the ~800 nucleotide band was not always visible (data not shown).

To map the regions of the GFnα-3 sequence responsible for protection of these RNAs, subclones were generated in M13mp19 that lacked various amounts of GFnα-3 5' DNA (Fig. 5*A*). One predicts that S1 nuclease digestion of heteroduplexes formed between RNA and any of these deleted clones will result in a smaller S1 protected fragment than when a full-length clone is used, if the RNA overlaps with the deleted region. When this experiment was carried out, all 3 larger protected bands decreased in size in proportion to the extent of the 5' deletion, indicating that these RNAs all span the 5' end of GFnα-3 and therefore must differ at their 3' ends (Fig. 5*B*). The largest (~2.7 kb) band represents full-length protection of the GFnα-3 sequence. The bands at 1.9 and 1.7 kb represent either (1) GFnα-3 gene products whose RNA transcripts contain shorter 3' untranslated regions than GFnα-3 or (2) sequences which diverge from the GFnα-3 sequence at their 3' ends. Consistent with the former possibility one finds 4 putative poly (A)-tail addition signal sequences in the GFnα-3 3' untranslated region (underlined in Fig. 1). These sites occur at nucleotides 1378, 1610, 2379, and 2444. If each of these sites were used during RNA processing, one predicts protected fragments of 2698, 2633, 1838, and 1606 nucleotides to result from the S1 experiment. The 2 largest fragments, differing by 65 nucleotides, would probably not be resolved on the agarose gel, making it difficult to determine if one of these sites is used preferentially. Nonetheless, the sizes of the protected fragments determined experimentally (Fig. 5*B*) agree fairly well with that predicted from alternative use of

```
                                                   1                          ◊                      50
GFnα-3   MKLQISGLLL VTAVAYATIE APEEFVSL   AEMEDTLLRN LFRGYQKWVR PILHANDTIT VRFGLKISQL VDVDEKNHLM
GFnα-2    MTLAVIGLF TLFTSIIAIT PAREFVSL   AEREDALLRE LFQGYQRWVR PVQHANHSVK VRFGLKISQL VDVDEKNQLM
Ratβ-3  MTGFLRVFLVL SATLSGSWVT LTATAGLSSV AEHEDALLRH LFQGYQKWVR PVLNSSDIIK VYFGLKISQL VDVDEKNQLM
Ratβ-2   MLACMAGHSN SMALFSFSLL WLCSGVLGTD TEERLVEHLL DPSRYNKLIR PATNGSELVT VQLMVSLAQL ISVHEREQIM
Ratβ-4  DARLFDCSGVL PDKGPAGLTV RFPGDCRLAN AEEKLMDDLL NKTRYNNLIR PATSSSQLIS IRLELSLSQL ISVNEREQIM
ARDnα        MESSCK SWLLCSILVL VAFSLVSASE DEERLVRDL. .FRGYNKLIR PVQNMTQKVG VRFGLAFVQL INVNEKNQVM
                                          *          *    * *              **   * *   *
         <-------SIGNAL PEPTIDE------->

                                                                              ◊        †
GFnα-3   TTNVWLWQEW TDYKLRWNPE DYGGITSIRV PSETIWLPDI VLYENADGRF EGSLMTKAIV RFNGTIMWTP PASYKSSCTM
GFnα-2   TTNVWLWQEW LDYKLRWNPE NYGGITSIRV PSESIWLPDI VLYENADGRF EGSLMTKAIV RYNGMITWTP PASYKSACTM
RATβ-3   TTNVWLKQEW TDQKLRWNPE EYGGINSIKV PSESLWLPDI VLFENADGRF EGSLMTKAIV KSSGTVSWTP PASYKSSCTM
Ratβ-2   TTNVWLTQEW EDYRLTWKPE DFDNMKKVRL PSKHIWLPDV VLYNNADGMY EVSFYSNAVV SYDGSIFWLP PAIYKSACKI
Ratβ-4   TTSIWLKQEW TDYRLAWNSS CYEGVNILRI PAKRVWLPDI VLYNNADGTY EVSVTYNVIV RSNGSIQWLP PAIYKSACKI
ARDnα    KSNVWLRLVW YDYQLQWDEA DYGGIGVLRL PPDKVWKPDI VLFNNADGNY EVRYKSNVLI YPTGEVLWVP PAIYQSSCTI
           * **   *  *  * *                *   * **  **  ****   *            *   * * ** * * *

                  ◊†                                      ◊                      206
GFnα-3   DVTFFPFDRQ NCSMKFGSWT YDGTMVDLTL ..LDAYVDRK DFFDNGEWEI LNATGQRGSR RDGIYS..YP YVTYSFILKR
GFnα-2   DVTFFPFDRQ NCSMKFGSWT YDGNMVKLVL ..INQQVDRS DFFDNGEWEI LSATGVKGSR QDSHLS..YP YITYSFILKR
Ratβ-3   DVTFFPFDRQ NCSMKFGSWT YDGTMVDLIL ..INENVDRK DFFDNGEWEI LNAKGMKGNR REGFYS..YP FVTYSFVLRR
Ratβ-2   EVKHFPFDQQ NCTMKFRSWT YDRTEIDLVL ..KSDVASLD DFTPSGEWDI IALPGRRNEN PDDST...YV DITYDFIIRR
Ratβ-4   EVKHFPFDQQ NCTLKFRSWT YDHTEIDMVL   KSPTAIMD DFTPSGEWDI VALPGRRTVN PQDPS    YV DVTYDFIIKR
ARDnα    DVTYFPFDQQ TCIMKFGSWT FNGDQVSLAL YNNKNFVDLS DYWKSGTWDI IEVPAYLNVY EGDSNHPTET DITFYIIIRR
          *  **** *  * *** ***        *         *   * * *                              *

                                  --  -+                 --      +      -
GFnα-3   LPLFYTLFLI IPCLGLSFLT VLVFYLPSDE GEKLLLSTSV LVSLTVFLLV IEEIIPSSSK VIPLIGEYLL FIMIFVTFSI
GFnα-2   LPLFYTLFLI IPCLGLSFLT VLVFYLPSDE GEKVSLSTSV LVSLTVFLLV IEEIIPSSSK VIPLIGEYLL FIMIFVTLSI
Ratβ-3   LPLFYTLFLI IPCLGLSFLT VLVFYLPSDE GEKLSLSTSV LVSLTVFLLV IEEIIPSSSK VIPLIGEYLL FIMIFVTLSI
Ratβ-2   KPLFYTINLI IPCVLITSLA ILVFYLPSDC GEKMTLCISV LLALTVFLLL ISKIVPPTSL DVPLVGKYLM FTMVLVTFSI
Ratβ-4   NALFYTINLI IPCVLITSLA ILVFYLPSDC GEKMTLCISV LLALTFFLLL ISKIVPP  L NIPLIGKYLL FTMVLVTFSI
ARDnα    KTLFYTVNLI LPTVLISFLC VLVFYLPAEA GEKVTLGISI LLSLVVFLLL VSKILPPTSL VLPLIAKYLL FTFIMNTVSI
          ** *  **  *       *  ******   ***  *  *  *  *  ***     * *       **   **  *     * **
         <----------TMD 1---------> -   -+ <-------TMD 2------>+               +<-----TMD 3---

                                                                                         366
GFnα-3   IVTLFVINVH HRSSATYHPM APWVKSLFLQ RLPRLLCMRG HTDRYQYPDI ELRSPELKRG MKKGQQKSAG GGRGGLKEDE
GFnα-2   IVTIFVINVH HRSSATYHPM SPWVRSLFLQ RLPHLLCMRG NTDRYHYPEL EPHSPDLKPR NKKGPPGPEG EGQALIN...
Ratβ-3   IVTVFVINVH HRSSSTYHPM APWVKRLFLQ RLPRWLCMKD PMDRFSFPDG KESDTAVRGK VSGKRKQTPA SDGERVLVAF
Ratβ-2   VTSVCVLNVH HRSPTT.HTM APWVKVVFLE KLPTLLFLQQ PRHRCARQRL RLRRRQRERE GEAVFFREGP AADPCTCFVN
Ratβ-4   VTTVCVLNVH HRSPST HTM ASWVKECFLH KLPTFLFMKR PGLEVSLVRV PHPSQLHLAT ADTAATSALG PTSPSNLYGS
ARDnα    LVTVIIINWN FRGPRT.HRM PMYIRSIFLH YLPAFLFMKR PRKTRLRWMM EMPGMSMPAH PHPSYGSPAE LPKHISAIGG
                   *     *    * * *         **    **   *
         -------->              <--------------------------------------------CYTOPLASMIC DOMAIN--

GFnα-3   NQAWIA.... .......... .......... .......... .......... LLEKATHSVH YISRHIKKEH FIREVVQDWK
GFnα-2   .......... .......... .......... .......... .......... LLEQATNSVR YISRHIKKEH FIREVVQDWK
Ratβ-3   .......... .......... .......... .......... .......... .LEKASESIR YISRHVKKEH FISQVVQDWK
Ratβ-2   PASVQGLAGA FRAEPTAAGP GRSVGPCSC. .......... .......... GLREAVDGVR FIADHMRSED DDQSVREDWK
Ratβ-4   SMYFVNPVPA APKSAVSSHT AGLPRDARLR SSGRFRE... .......... DLQEALEGVS FIAQHLESDD RDQSVIEDWK
ARDnα    KQSKMEVMEL SDLHHPNCKI NRKVNSGGEL GLGDGCRRES ESSDSILLSP EASKATEAVE FIAEHLRNED LYIQTREDWK
                                                                *         *  *              ***
         ----------------------------------------------------------->

                                 433
GFnα-3   FVAQVLDRIF LWVFLTASVL GTILIFTPAL HMYLST.... ..........
GFnα-2   FVAQVLDRIF LWTFLTVSVL GTILIFTPAL KMFLRTPPPPSP.......
Ratβ-3   FVAQVLDRIF LWLFLIASVL GSILIFIPAL KMWIHRFH.. ..........
Ratβ-2   YVAMVIDRLF LWIFVFVCVF GTVGMFLQPL FQNYTATTFL HPDHSAPSSK
Ratβ-4   FVAMVVDRLF LWVFLFVCIL GRTMGLFLPP LFQIHAPSKD S
ARDnα    YVAMVIDRLQ LYIFFIVTTA GTVGILMDAP HIFEYVDQDR IIEIYRGK..
          ** * **    *  *      *
               <-------TMD 4------->
```

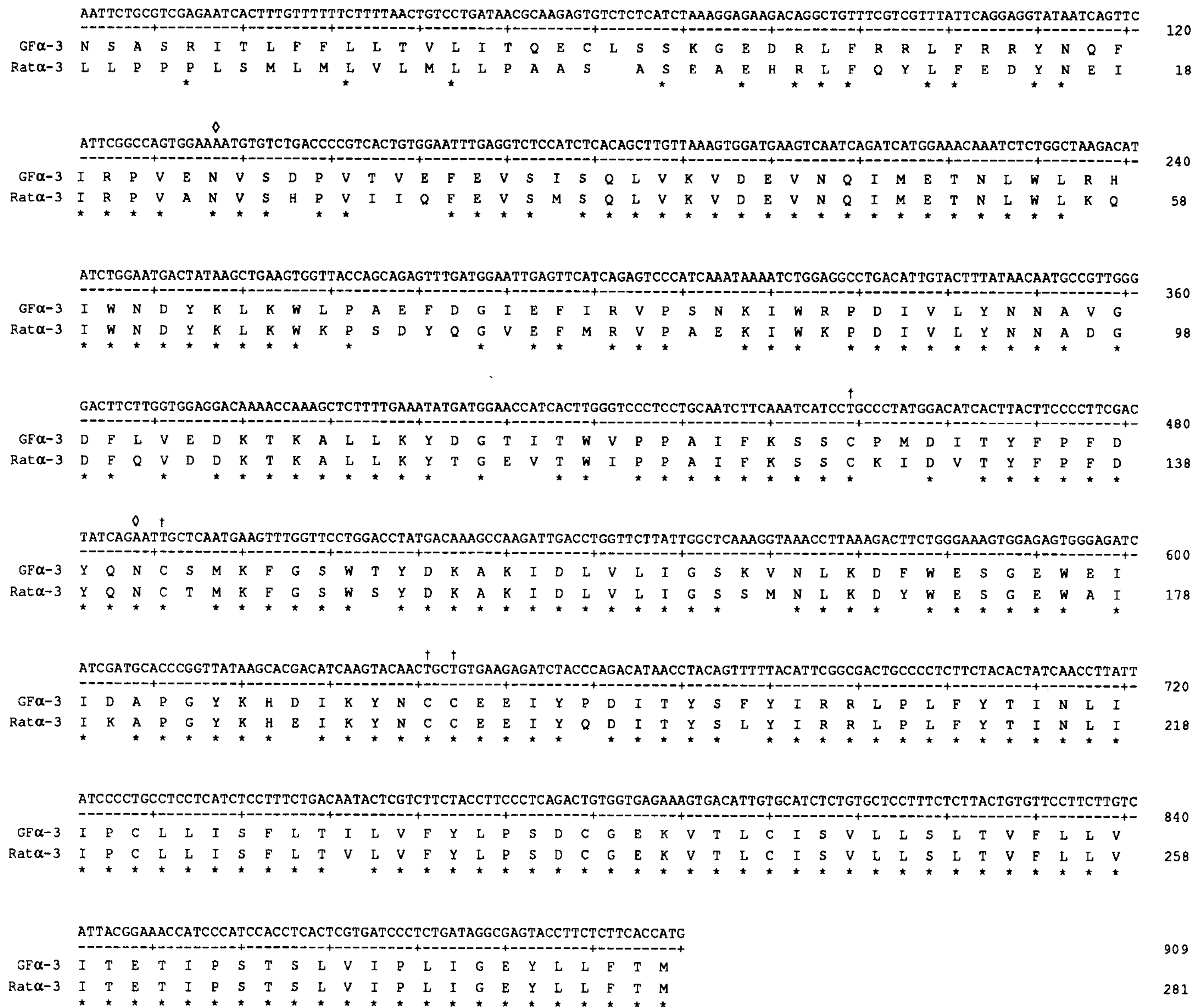


Figure 3. Nucleotide and deduced amino acid sequence of cDNA clone GFα-3, aligned with the rat α-3 amino acid sequence (Boulter et al., 1986). Amino acids conserved in both subunits are indicated by an *asterisk. Diamonds* indicate potential glycosylation sites and *daggers* indicate conserved cysteine residues.

poly(A) addition sites based on DNA sequence analysis (Figs. 1, 5*C*). Since Northern blots only detected hybridizing RNA at about 2.7 kb, it is likely that these smaller S1 nuclease-resistant fragments have longer 5' ends than clone GFnα-3.

The smallest fragment observed in these S1 nuclease protection experiments was approximately 0.8 kb. Its intensity and appearance was heterogeneous, appearing clearly in 2 out of 5 experiments. S1 nuclease protection experiments using sense and antisense single-stranded DNAs from GFnα-2 and GFnα-3 cDNAs show that this ~800 bp fragment does not result from hybridization between these very similar gene products. Therefore, this fragment may represent the product of a gene related to GFnα-3 (but not GFnα-2) or alternative splicing of the GFnα-3 gene primary transcript.

GFα-3 identifies a single RNA transcript expressed in retina and brain

To determine the number of RNAs in retina and brain that are identified by the GFα-3 cDNA, S1 nuclease protection experiments were performed with this clone (Fig. 6). The GFα-3 cDNA

←

Figure 2. Comparison of deduced amino acid sequences for neural nicotinic ACh receptor non-alpha (nα) subunits. Shown are nα subunit sequences from *Drosophila* ARDnα; (Hermans-Borgmeyer et al., 1986), rat β-2 and β-3 (Deneris et al., 1988, 1989), rat β-4 (clone SCG3, Isenberg and Meyer, 1989) and goldfish (GFnα-2 and GFnα-3; Cauley et al., 1989). Amino acids conserved in all 6 subunits are indicated by an *asterisk.* The putative transmembrane and cytoplasmic domains are indicated below the aligned sequences. *Diamonds* indicate potential glycosylation sites for GFnα-3, and *daggers* indicate conserved cysteine residues. *Minus* and *plus* symbols refer to charged amino acid residues appearing on either side of TMD 2 (see Discussion).

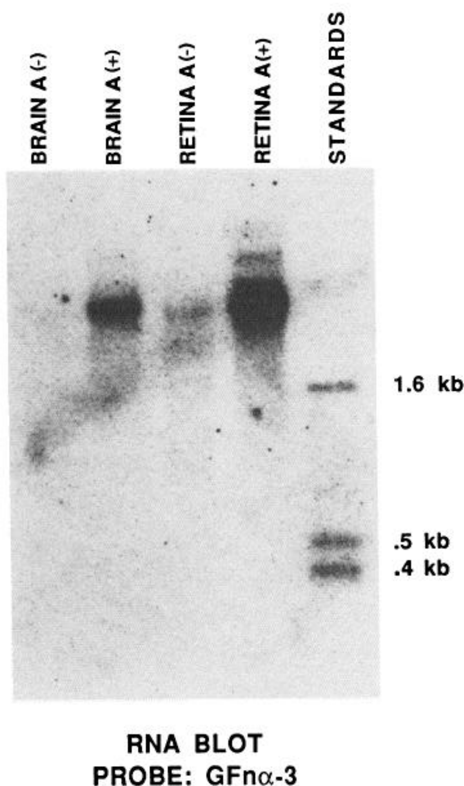


Figure 4. GFnα-3 identifies multiple RNAs in retina and brain. Northern blot analysis of RNAs homologous to the GFnα-3 cDNA. 5 μg of poly (A)+ RNA from brain (lane 2), 2.5 μg of poly(A)+ RNA from retina (lane 4) and 10 μg of poly(A)− RNA from brain (lane 1) and retina (lane 3) was size fractionated on a 2.2 M formaldehyde 1.2% agarose gel and transferred to Gene Screen Plus membrane. The blot was probed with ^{32}P-labeled GFnα-3 cDNA, and washed at low stringency.

was subcloned into M13mp19 to generate single-stranded DNA. Heteroduplexes were formed between this DNA and poly(A)+ RNA isolated from goldfish brain and retina. Heteroduplexes were then digested with S1 nuclease, and S1-resistant fragments were fractionated on a denaturing agarose gel. This analysis revealed a single protected fragment of ~900 bp when either retina or brain RNA was used (Fig. 6). This is the expected size for complete protection of the cDNA by the RNA. In addition, densitometry of the autoradiogram indicates that the relative level of expression of the GFα-3 gene is about 4-fold higher in retina than brain. Since GFα-3 is only a partial cDNA, it was not possible to determine if other RNAs exist that are similar to the GFα-3 gene product but diverge in sequence at their 3′ ends. The small amount of signal seen in the *control* lane in Figure 6 is not reproducible and could represent overflow from neighboring wells or residual undigested M13 DNA.

In situ *hybridization identifies cells in retina and tectum expressing nAChR genes*

To date we have isolated and characterized 3 different genes expressed in goldfish retina: (1) GFnα-2 (Cauley et al., 1989), (2) GFnα-3, and (3) GFα-3. In order to determine if these genes are expressed in similar cell types, we have compared their pattern of expression in retina and tectum using the high-resolution technique of *in situ* hybridization. In retina, these experiments showed all 3 genes to be expressed in the retinal ganglion cell layer (Fig. 7). In addition, the level of expression of these genes was heterogeneous among individual cells of this layer. Analysis of propidium iodide fluorescently back-labeled retinal sections showed that these cells are ganglion cells (data not shown), and we calculate that greater than 70% of the ganglion cells hybridize positively for each probe. Further study of this heterogeneity in *in situ* hybridization among the ganglion cells is in progress. Besides being expressed by ganglion cells, the GFnα-3 gene is expressed at high levels, relative to GFnα-2 and GFα-3, in the inner nuclear layer of the retina. Sections were examined before staining to verify that emulsion grains resulting from hybridization were not obscured by the histological stains used to visualize individual cells in the ganglion cell layer.

Since nAChRs are synthesized by retinal ganglion cells and transported to the optic tectum (Henley et al., 1986a; Sargent et al., 1987), we were interested in determining if cells in the tectum also express nAChR genes. In the optic tectum *in situ* hybridization identifies cells that express the GFnα-3 and GFα-3 genes; however no detectable expression of the GFnα-2 gene was observed (Fig. 8). Both GFnα-3 and GFα-3 probes hybridize to cells of the deepest, and most cell-dense layer, of the optic tectum. This layer can be identified as layer 3 of the periventricular zone (PVZ; Northcutt, 1983). In addition, positively hybridizing cells are scattered throughout the more superficial tectal layers (Fig. 8*C*). S1 nuclease protection experiments using single-stranded antisense GFnα-3 cDNA and tectal RNA showed the same multiplicity of protected fragments as observed when retinal RNA was used (data not shown). This result indicates similar processing of the GFnα-3 primary transcripts in tectum as in retina. Additional *in situ* hybridization signal can be seen in lower brain areas, with positive hybridization over the nucleus diffusus (DF), and strong hybridization over cells in the vicinity of the nucleus lateralis thalami (LT; Braford and Northcutt, 1983).

Discussion

We had previously identified a novel structural subunit (GFnα-2) expressed in the goldfish retina (Cauley et al., 1989). Here we report the identification of a second novel goldfish nAChR structural subunit, GFnα-3, and a putative ligand-binding α-subunit, GFα-3. Like GFnα-2, GFnα-3 encodes a protein classified as a non-alpha (nα)-like subunit of the nAChR because it lacks the adjacent cysteine residues corresponding to amino acids 192 and 193 of the *Torpedo* nAChR α-subunit (Noda et al., 1982). These residues correspond to a region of the α-subunit that contributes to the ACh binding site (Kao and Karlin, 1986).

GFnα-2 and GFnα-3 are the most homologous nAChR subunits identified to date, possessing 88% amino acid sequence similarity (Table 1). In addition, both of these sequences bear a striking similarity to the rat β3 sequence (Deneris et al., 1989; 82 and 85% amino acid sequence similarity, respectively; Table 1). Based on this similarity, it is possible that either GFnα-2 or GFnα-3 represents the goldfish homolog of the rat β3 gene. Unlike GFnα-2 and the rat β-3 sequence, GFnα-3 possesses 2 putative N-glycosylation sites, at positions 113 and 180 (Fig. 2). Posttranslational modifications might contribute to greater differences between the protein products of these genes than amino acid sequence homology would predict. Based on this hypothesis, and in lieu of additional data about the expression of these genes or the properties of their encoded proteins, it is

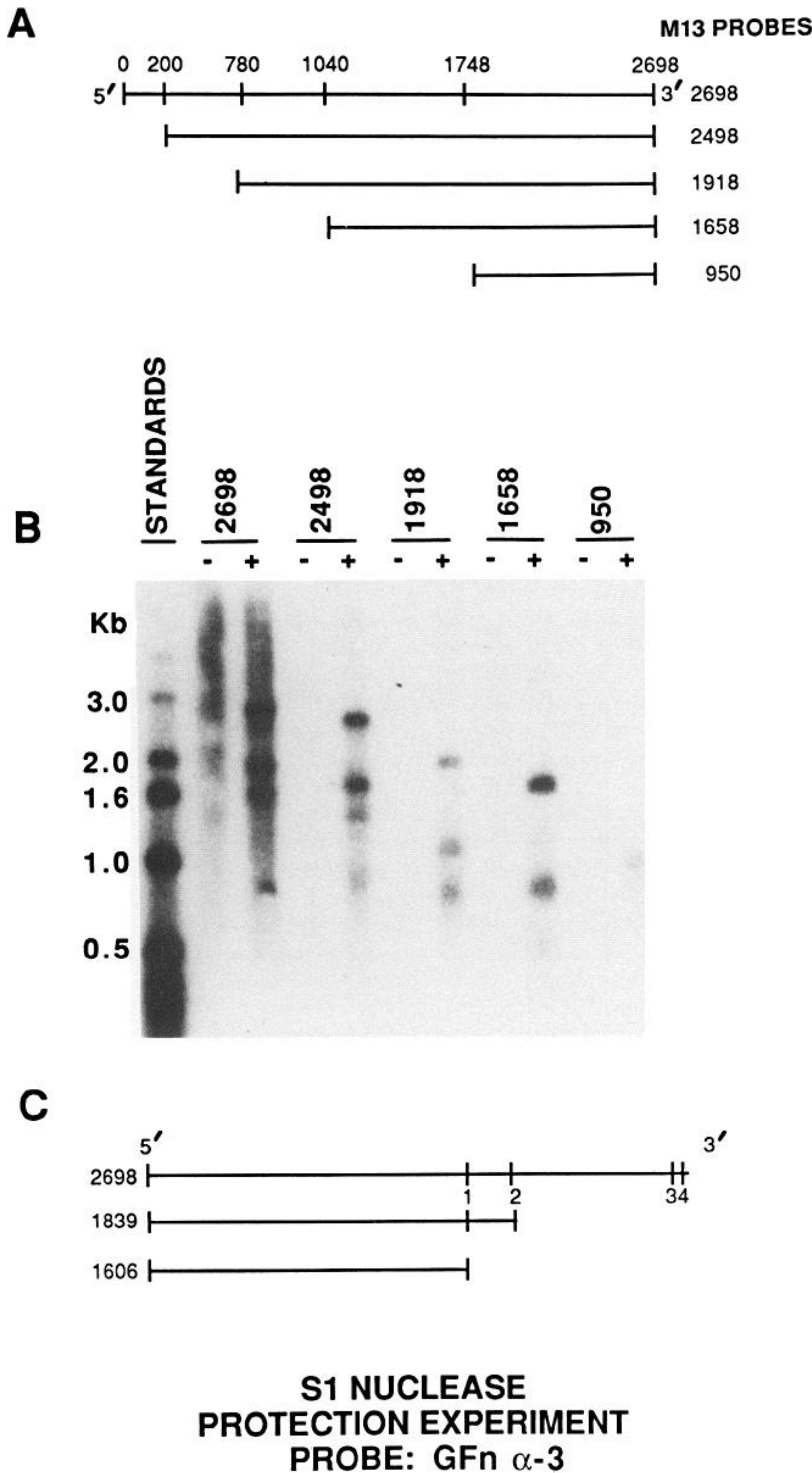


Figure 5. GFnα-3 identifies 3 retinal RNAs differing in their 3′ untranslated sequence. S1 nuclease protection experiment with GFnα-3 cDNA and retinal RNA. *A,* Line diagram of GFnα-3 M13 subclones used to hybridize with retinal RNA. The full-length clone is 2698 bases long. Deletions of the 5′ end were generated, and these M13 subclones were named according to the number of nucleotides remaining at the 3′ end. *B,* Gel profile of S1 nuclease protected fragments generated by S1 nuclease digestion of heteroduplexes formed between 5 μg poly(A)+ RNA isolated from retinal tissue and the M13 probes shown in *A.* (+) lanes contain RNA and (−) lanes are controls lacking RNA. The 4 bands in lane 2698+ are ~2.7, 1.9, 1.7, and 0.8 kb. *C,* Line diagram illustrating putative polyadenylation sites of the GFnα-3, and corresponding length of the mRNA that would be protected by the full-length GFnα-3 cDNA. These potential polyadenylation sites may account for the 3 larger bands generated by the S1 protection experiment (*B,* lane 2698+).

probable that GFnα-2 is more similar to the rat β3 gene. Assuming that the rat β3 gene is the rat homolog of either the goldfish GFnα-2 or GFnα-3 gene, and including as well the *Drosophila* ARDnα subunit, the identification of GFnα-3 brings the total number of neural nAChR structural subunits identified to date to 5 (Fig. 2, Table 1).

Recently, site-directed mutagenesis has identified charged residues surrounding transmembrane domain 2 (TMD 2) of each subunit of the *Torpedo* nAChR as influencing ion flow through the channel (Imoto et al., 1988). Analysis of this region in the neural non-alpha subunits indicates 2 general classes. One class, comprising GFnα-3, GFnα-2, and the rat β-3 gene, has 3 negatively charged residues (Asp 235, Glu 236, Glu 238) and 1 positively charged residue (Lys 239) on the putative cytoplasmic

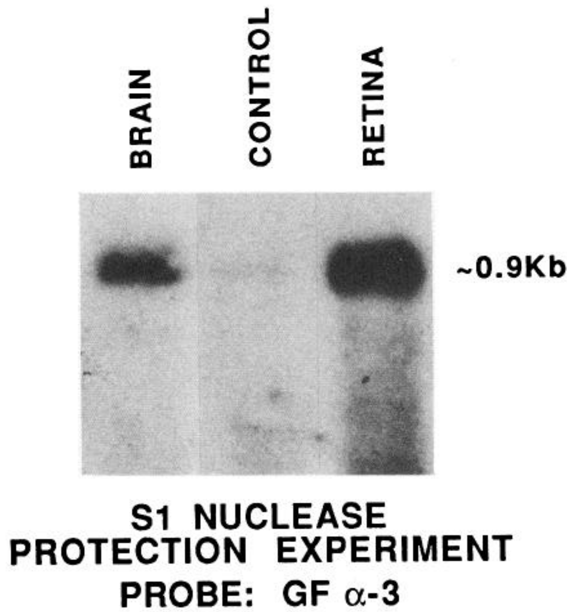


Figure 6. GFα-3 identifies a single RNA in retina and brain. S1 nuclease protection experiment with GFα-3 cDNA and retina or brain RNA. S1 nuclease protected fragments generated from brain (lane 1) and retinal (lane 3) RNA are both ~900 bases in length. Control protection (lane 2) was carried out using a sense orientation GFα-3 M13 subclone.

side of TMD 2 (Fig. 2). On the extracellular side of TMD 2, these subunits share 3 negatively charged residues (Glu 258, Glu 259, Glu 273) and 1 positively charged amino acid (Lys 266). The second class, represented by clones rat β2 (Deneris et al., 1988), ARDnα (Hermans-Borgmeyer et al., 1986), and rat β4 (clone SCG 3; Isenberg and Meyer, 1989) contain 2 negatively and 1 positively charged residue (Asp or Glu 235, Glu 238, Lys 239) on the cytoplasmic side and 2 positively charged residues (Lys 259, Lys 273) on the extracellular side of TMD 2 (Fig. 2). The neural α- subunits each possess 2 negatively (Asp or Glu) and 1 positively charged (Lys) residue on the cytoplasmic side and 2 negatively charged residues (Glu) on the extracellular side of TMD 2. For the *Torpedo* nAChR subunits, channel conductance is proportional to the net number of negative charges neighboring the hydrophobic segment TMD 2 (Imoto et al., 1988). One can therefore predict that the nAChRs using the GFnα-3 class of subunits have a greater conductance than those of the rat β2 class. Expression and electrophysiological recording studies are necessary to confirm this hypothesis.

Since GFnα-2 and GFnα-3 represent novel members of the nAChR gene family, it was important to determine whether similar nAChR genes are expressed in fish and higher vertebrates. Clearly the mammalian muscle type of nAChR is expressed in the electric organ of the electric fish (Boulter et al., 1985). In addition, we determined that other known members of the mammalian nAChR gene family were represented in our goldfish retinal cDNA library. From our original library screening we identified a partial cDNA (GFα-3) with 89% amino acid sequence similarity to the rat α-3 sequence (Fig. 3). GFα-3 contains the double cysteine residues corresponding to positions 192 and 193 of the *Torpedo* α-subunit (Noda et al., 1982) consistent with its classification as an α-subunit. An 89% similarity between the GFα-3 and the rat α3 gene product suggests that they may be homologous genes. Comparison of the known homologous rat and chick nAChR gene products indicates similarities of 81% for the α-4 polypeptide, 89% for the β-2 polypeptide, and 92% for the α-3 polypeptide (rat α-3 Boulter et al., 1986; rat α-4, Goldman et al., 1987; rat β-2 Deneris et al., 1988; chicken α-3, α-4, β-2, Nef et al., 1988). Therefore, GFα-3 most likely represents the goldfish homolog of the rat and chick α-3 cDNAs and demonstrates that similar members of the nAChR gene family are expressed in these 3 species. Also, as previously mentioned, the rat β-3 gene may represent the rat homolog of either the goldfish GFnα-2 or GFnα-3 genes.

The level of GFα-3 and GFnα-3 gene expression in goldfish retina and brain was investigated by Northern blot analysis and S1 nuclease protection experiments. Both genes are expressed in retina and in brain (Figs. 4–6). This is in contrast to the GFnα-2 gene, whose expression was not detected in 5 μg of poly(A)+ brain RNA (Cauley et al., 1989). In addition, GFnα-3 gene expression results in synthesis of multiple RNAs most likely generated by alternative use of polyadenylation signal sequences (Fig. 5).

In situ hybridization was used to determine which cells in the goldfish retina express the GFnα-3 and GFα-3 genes and to compare this expression to that seen for the GFnα-2 gene. All 3 genes are expressed in the ganglion cell and inner nuclear layer of the goldfish retina (Fig. 7). However, unlike GFnα-2 and GFα-3, GFnα-3 is expressed at a significantly higher level in the inner nuclear layer than in the ganglion cell layer (Fig. 7). Hybridization of these probes to cells of the inner nuclear layer is not due to displaced ganglion cells, as only a few percent of ganglion cells are displaced in the goldfish retina (Hitchcock and Easter, 1986; Tumosa and Stell, 1986). Consistent with these results, Keyser et al. (1988) and Sargent et al. (1989) report nAChR-like immunoreactivity in the inner nuclear layer and ganglion cell layer of chick and frog retina, respectively.

For each probe, hybridization in the retinal ganglion cell layer is heterogeneous. Some ganglion cells (established by fluorescent backfilling) express the nAChR genes at relatively high levels, while others express these genes at low or undetectable levels. Whether this reflects a functional difference between ganglion cells is not clear. Our observations are consistent with previous reports that nonganglion cell neuronal somata in the ganglion cell layer are infrequent occurrences in the goldfish retina (Hitchcock and Easter, 1986; Tumosa and Stell, 1986).

The retina's inner nuclear layer is comprised primarily of amacrine, horizontal and bipolar neurons (Dowling, 1987). α-BTX binding sites are reported to be expressed by bipolar cells in the turtle retina (James and Klein, 1985) and by amacrine, bipolar, and ganglion cells in the goldfish retina (Schwartz et al., 1980; Zucker and Yazulla, 1982; Henley et al., 1986a). Most of these sites appear to be extrasynaptic (Zucker and Yazulla, 1982). The cholinergic amacrine cell appears to be the only cholinergic neuron in the retina (Masland and Mills, 1979; Tumosa and Stell, 1986). Though electron microscopy studies suggest that cholinergic amacrine cells synapse only upon ganglion cells (Famiglietti, 1983), a more recent study reports synapses between cholinergic amacrine cells (Millar and Morgan, 1987). Therefore, the expression of nAChRs genes by cells of the inner nuclear layer may reflect the synthesis of (1) extrasynaptic receptors on amacrine, horizontal, and/or bipolar cells; (2) presynaptic receptors; or (3) receptors synthesized by cells of the inner nuclear layer that are postsynaptic to cholinergic amacrine cell input. GFnα-2 shows preferential hybridization

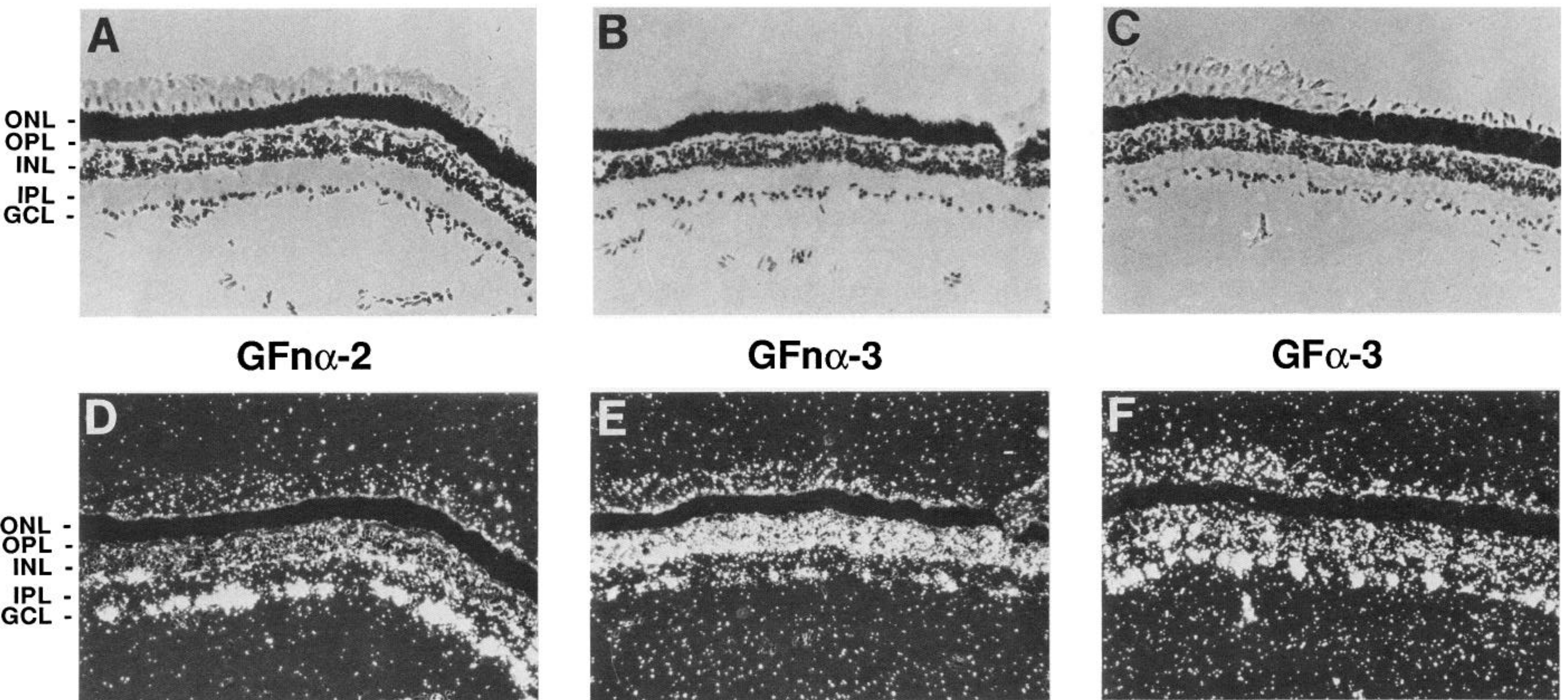
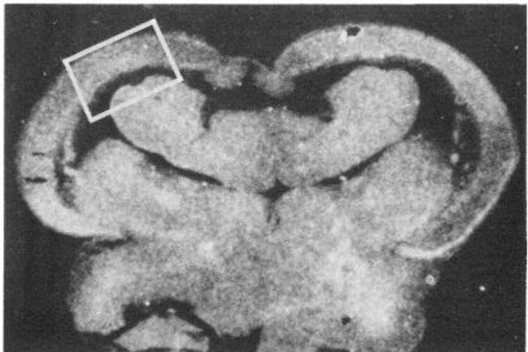
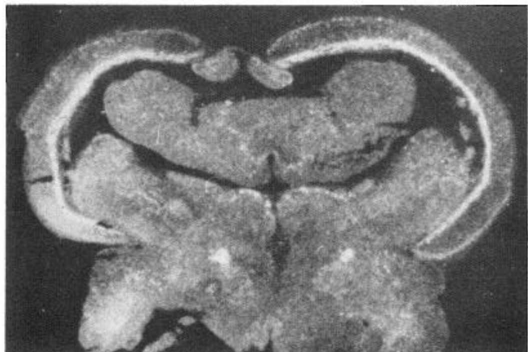
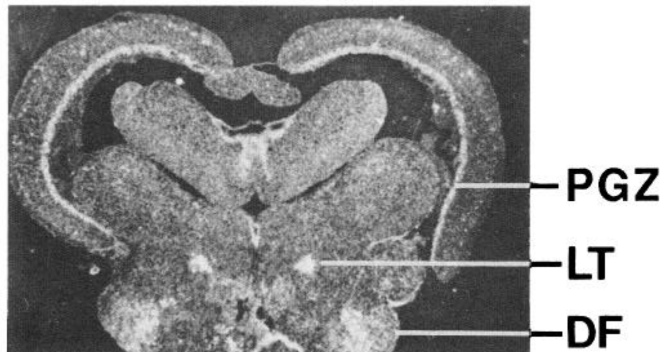


Figure 7. nAChR gene expression in goldfish retina. *In situ* hybridizations of GFnα-2, GFnα-3 and GFα-3 to sections of goldfish retina. Goldfish retinal sections were hybridized with ^{35}S-labeled RNA corresponding to GFnα-2, GFnα-3 or GFα-3 in the antisense orientation. *A, B,* and *C* are brightfield photographs of hematoxylin and eosin stained sections, while *D, E,* and *F* are the same sections photographed with darkfield illumination, 20X objective. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.



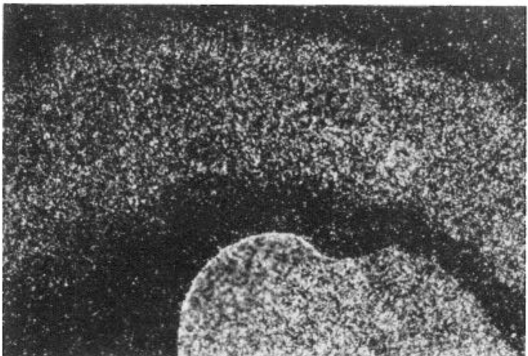


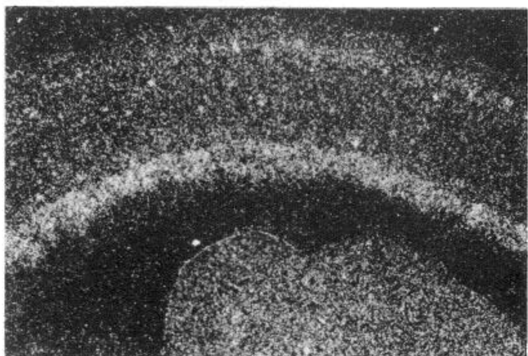


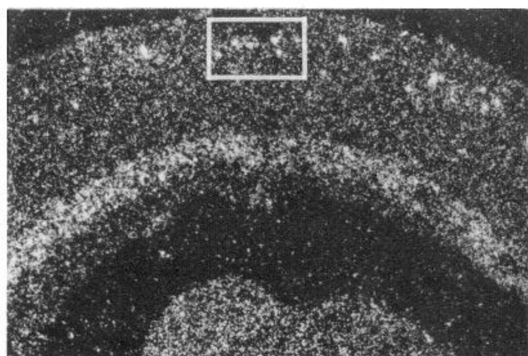
GFnα-2

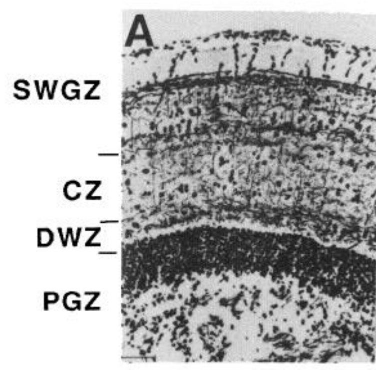
GFnα-3

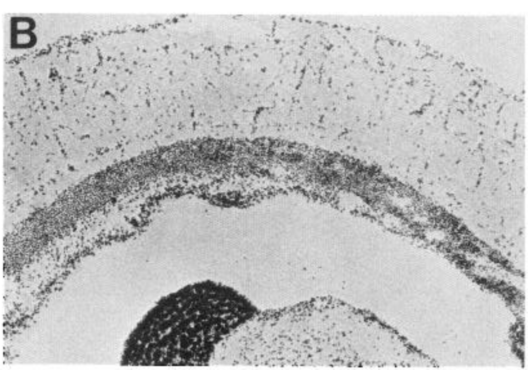
GFα-3

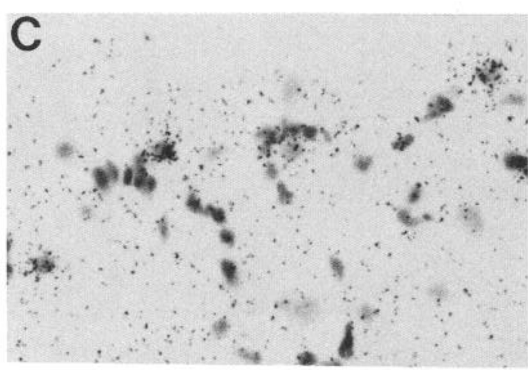












along the inner margin of the inner nuclear layer, where amacrine cells reside (Dowling, 1987), and so might be more amacrine cell-specific than GFnα-3, which shows hybridization throughout the inner nuclear layer (Fig. 7, *D, E*). These *in situ* data establish that the goldfish retina expresses multiple members of the nAChR gene family and suggest that the vertebrate retina utilizes more than one type of nicotinic cholinergic synapse.

The retinotectal synapse of lower vertebrates is one of the most approachable synapses of the vertebrate CNS (Oswald and Freeman, 1980). nAChRs play a role, albeit yet unclear, in retinotectal synapse function (Schmidt and Freeman, 1980; Langdon and Freeman, 1987). A nAChR-like molecule is synthesized by retinal ganglion cells and transported to the optic tectum in goldfish (Henley et al., 1986a). Pharmacological studies support the notion that the principal neurotransmitter of the retinotectal synapse is an excitatory amino acid (Langdon and Freeman, 1986). The optic neuropil of the tectum, however, receives a cholinergic input from a tegmental visual nucleus, the nucleus isthmi (Ricciuti and Gruberg, 1985). The demonstration of nAChRs on ganglion cell afferents has led to the proposal that ACh release by nucleus isthmi inputs somehow regulates glutamate release from optic axon terminals (Sargent et al., 1989). Such appears to be the role of ACh in the nigrastriatal pathway, where dopamine release from striatal terminals can be modulated by presynaptic nAChRs (Giorguieff-Chesselet et al., 1979; Clark and Pert, 1985). To further elucidate the type and localization of nAChRs in the optic tectum, we performed *in situ* hybridization studies in optic tectum with the 3 cDNA clones GFnα-2, GFnα-3, and GFα-3. These experiments show relatively high levels of expression of the GFnα-3 and GFα-3 genes in optic tectum (Fig. 8), while tectal GFnα-2 gene expression was not detected. Both GFnα-3 and GFα-3 probes show hybridization over cells of layer 3 of the PVZ, and cells scattered throughout the more superficial tectal layers (Fig. 8; Northcutt, 1983).

The identification of cells in the optic tectum expressing nAChR genes shows for the first time that tectal cells have the potential to synthesize nAChRs. Choline acetyltransferase (ChAT) staining in tectum reveals a single population of intrinsic tectal neurons that are cholinergic. These are a subpopulation of the abundant type XIV neurons of the PVZ (Tumosa et al., 1986). These neurons send apical dendrites into the more superficial tectal layers, where optic nerve inputs are found (Ross and Godfrey, 1986; Tumosa et al., 1986). However, the majority of tectal ChAT activity seems to come from tectal inputs originating from the nucleus isthmi (Ricciuti and Gruberg, 1985). It therefore seems likely that nAChRs expressed by cells of the PVZ and more superficial layers (represented by clones GFnα-3 and GFα-3) respond to cholinergic inputs from one or both of these sources. These data indicate that putative nAChRs in the optic tectum result from gene expression of cells both extrinsic (retinal ganglion cell; Henley et al., 1986a) and intrinsic to this area of the visual forebrain.

In conclusion, we have presented the isolation and characterization of 2 members of the nAChR gene family, expressed in goldfish retina and tectum. GFnα-3 represents a new gene, encoding a nAChR structural subunit. GFnα-3 is highly homologous to the previously identified GFnα-2 and rat β-3 genes. In spite of their sequence similarity, the pattern of expression of the GFnα-2 and GFnα-3 genes in retina and brain is quite different, indicating that they participate in different receptor systems. We have also characterized the expression of a partial cDNA clone, GFα-3, encoding an agonist binding subunit likely to represent the goldfish homolog of the rat and chicken α-3 genes. *In situ* hybridization with these clones evidences the expression of multiple nAChR genes in retina and tectum.

References

Ames, A., III, and D. A. Pollen (1968) Neurotransmission in central nervous tissue: A study of isolated rabbit retina. J. Neurophysiol. *32:* 424–442.

Ariel, M., and N. W. Daw (1982) Effects of cholinergic drugs on receptive field properties of rabbit retinal ganglion cells. J. Physiol. (Lond.) *324:* 135–160.

Aviv, H., and P. Leder (1972) Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA *69:* 1408–1412.

Barnard, E. A., M. G. Darlison, and P. Seeburg (1987) Molecular biology of the $GABA_A$ receptor: The receptor/channel superfamily. Trends Neurosci. *10:* 502–509.

Betz, H., and U. Müller (1981) Characterization of the α-bungarotoxin receptor in chick embryo retina. Eur. J. Biochem. *117:* 131–139.

Birnstiel, M. L., M. Busslinger, and K. Strub (1985) Translation termination and 3′ processing: The end is in site! Cell *41:* 349–359.

Boulter J., W. Luyten, K. Evans, P. Mason, M. Ballivet, D. Goldman, S. Stengelin, G. Martin, S. Heinemann, and J. Patrick (1985) Isolation of a clone coding for the α-subunit of a mouse acetylcholine receptor. J. Neurosci. *5:* 2545–2552.

Boulter, J., K. Evans, D. Goldman, G. Martin, D. Treco, S. Heinemann, and J. Patrick (1986) Isolation of a cDNA clone coding for a possible neural nicotinic acetylcholine receptor α-subunit. Nature *319:* 368–374.

Braford, M. R., Jr., and R. G. Northcutt (1983) Organization of the diencephalon and pretectum of the ray-finned fishes. In *Fish Neurobiology, Vol. 2: Higher Brain Areas and Functions,* R. E. Davis and R. G. Northcutt, eds., p. 158, University of Michigan Press, Ann Arbor.

Cauley, K., B. W. Agranoff, and D. Goldman (1989) Identification of a novel nicotinic acetylcholine receptor structural subunit expressed in goldfish retina. J. Cell Biol. *108:* 637–645.

Changeux, J. P. (1980) The acetylcholine receptor: An "allosteric" membrane protein. Harvey Lectures *75:* 85–254.

Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry *18:* 5294–5299.

←

Figure 8. nAChR gene expression in goldfish brain. *In situ* hybridizations of GFnα-2, GFnα-3 and GFα-3 to sections of goldfish brain. Coronal sections cut through the optic tectal lobes were hybridized with ^{35}S-labeled RNA corresponding to GFnα-2, GFnα-3 or GFα-3 in the antisense orientation. Below each coronal section is a 10× magnification of optic tectum from a region corresponding to the boxed area of the GFnα-2 section. All 6 figures are darkfield illumination. Areas showing positive hybridization for GFnα-3 and GFα-3 include the periventricular gray zone (PGZ) and scattered cells of more superficial tectal layers, the vicinity of the nucleus lateralis thalami (LT), and the nucleus diffusus (DF; Braford and Northcutt, 1983). *A, lower left,* Illustrates goldfish tectal anatomy. SWGZ, superficial white and grey zone; CZ, central zone; DWZ, deep white zone; PGZ periventricular grey zone (Northcutt, 1983). *B, lower center,* GFnα-3 probed tectal section, stained with hematoxylin and eosin, 10× magnification and brightfield illumination. *C, lower right,* GFα-3 hybridizing cells in superficial layer of optic tectum at 40× magnification. This region corresponds to the boxed area of the GFα-3 10× magnification photograph (directly above) and illustrates GFα-3 gene expression by cells of the tectal superficial white and grey zone.

Clark, P. B. S., and A. Pert (1985) Autoradiographic evidence for nicotine receptors on nigrastriatal and mesolimbic dopaminergic neurons. Brain Res. *348:* 355–358.

Clark, P. B. S., R. D. Schwartz, S. M. Paul, C. B. Pert, and A. Pert (1985) Nicotine binding in the rat brain: Autoradiographic comparison of [^{3}H] acetylcholine, [^{3}H] nicotine and [^{125}I] alpha-bungarotoxin. J. Neurosci. *5:* 1307–1315.

Cox, K. H., D. V. DeLeon, L. M. Angerer, and R. C. Angerer (1984) Detection of mRNAs in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. Dev. Biol. *101:* 485–502.

Dale, R. M. K., B. A. McClure, and J. P. Houchins (1985) A rapid single-stranded cloning strategy for producing a sequential series of overlapping clones for use in DNA sequencing: Application to sequencing the corn mitochondrial 18S rRNA. Plasmids *13:* 31–40.

Deneris, E. S., J. Connolly, J. Boulter, E. Wada, K. Wada, L. W. Swanson, J. Patrick, and S. Heinemann (1988) Primary structure and expression of $\beta 2$: A novel subunit of neuronal nicotinic acetylcholine receptors. Neuron *1:* 45–54.

Deneris, E. S., J. Boulter, L. W. Swanson, J. Patrick, and S. Heinemann (1989) $\beta 3$: A new member of nicotinic acetylcholine receptor gene family is expressed in brain. J. Biol. Chem. *264:* 6268–6272.

Dowling, J. E. (1987) *The Retina: An Approachable Part of the Brain,* Belknap Press of Harvard U. P., Cambridge, MA.

Famiglietti, E. (1983) On and off pathways through amacrine cells in the mammalian retina: The synaptic connections of "starburst" amacrine cells. Vision Res. *23:* 1265–1279.

Fontaine, B., M. B. Sassoon, and J.-P. Changeux (1988) Detection of the nicotinic acetylcholine receptor α-subunit mRNA by *in situ* hybridization at neuromuscular junctions of 15-day-old chick striated muscles. EMBO J. *7:* 603–609.

Francis, C. M. (1953) Cholinesterase in the retina. J. Physiol. (Lond.) *120:* 435–439.

Giorguieff-Chesselet, M. F., M. L. Kemel, D. Wandscheer, and J. Glowinski (1979) Regulation of dopamine release by presynaptic nicotinic receptors in rat striatal slices: Effect of nicotine in a low concentration. Life Sci. *25:* 1257–1262.

Glickman, R. D., A. R. Adolph, and J. E. Dowling (1982) Inner plexiform circuits in the carp retina: Effects of cholinergic agonists, gaba, and substance P on the ganglion cells. Brain Res. *234:* 81–99.

Goldman, D., J. Boulter, S. Heinemann, and J. Patrick (1985) Muscle denervation increases the levels of two mRNAs coding for the acetylcholine receptor alpha-subunit. J. Neurosci. *5:* 2553–2558.

Goldman, D., E. Deneris, W. Luyten, A. Kochhar, J. Patrick, and S. Heinemann (1987) Members of a nicotinic acetylcholine receptor gene family are expressed in different regions of the mammalian central nervous system. Cell *48:* 965–973.

Graham, L. T. (1974) Comparative aspects of neurotransmitters in the retina. In *The Eye, Comparative Physiology,* Vol. 6, H. Davson and L. T. Graham, eds., pp. 283–342, Academic, New York.

Heacock, A. M., and B. W. Agranoff (1982) Protein synthesis and transport in the regenerating goldfish visual system. Neurochem. Res. *7:* 771–788.

Henley, J. M., and R. E. Oswald (1987) Two distinct (−) nicotine binding sites in goldfish brain. J. Biol. Chem. *262:* 6691–6698.

Henley, J. M., J. M. Lindstrom, and R. E. Oswald (1986a) Acetylcholine receptor synthesis in retina and transport to optic tectum in goldfish. Science *232:* 1627–1629.

Henley, J. M., M. Mynlieff, J. M. Lindstrom, and R. E. Oswald (1986b) Interaction of monoclonal antibodies to electroplaque acetylcholine receptors with α-bungarotoxin binding site of goldfish brain. Brain Res. *364:* 405–408.

Hermans-Borgmeyer, I., D. Kopf, R.-P. Ryseck, B. Hovemann, H. Betz, and E. D. Gundelfinger (1986) Primary structure of a developmentally regulated nicotinic acetylcholine receptor protein from Drosophila. EMBO J. *5:* 1503–1508.

Hitchcock, P. F., and S. S. Easter, Jr. (1986) Retinal ganglion cells in goldfish: A qualitative classification into four morphological types, and a quantitative study of the development of one of them. J. Neurosci. *6:* 1037–1050.

Imoto, K., C. Busch, B. Sakmann, M. Mashina, T. Konno, J. Nakai, H. Bujo, Y. Mori, K. Fukuda, and S. Numa (1988) Rings of negatively charged amino acids determine the acetylcholine receptor channel conductance. Nature *335:* 645–648.

Isenberg, K. E., and G. E. Meyer (1989) Cloning of a putative neuronal nicotinic acetylcholine receptor subunit. J. Neurochem. *52:* 988–991.

James, W. M., and W. L. Klein (1985) α-Bungarotoxin receptors on neurons isolated from turtle retina: Molecular heterogeneity of bipolar cells. J. Neurosci. *5:* 352–361.

Kao, P. N., and A. Karlin (1986) Acetylcholine receptor binding site contains a disulfide cross-link between adjacent half-cystinyl residues. J. Biol. Chem. *261:* 8085–8088.

Keyser, K. T., T. E. Hughes, P. J. Whiting, J. M. Lindstrom, and H. J. Karten (1988) Cholinergic neurons in the retina of the chick: An immunohistochemical study of the nicotinic acetylcholine receptors. Vis. Neurosci. *1:* 349–366.

Kozak, M. (1989) The scanning model for translation: An update. J. Cell Biol. *108:* 229–241.

Kyte, J., and R. F. Doolittle (1982) A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. *1:* 105–132.

Langdon, R. B., and J. A. Freeman (1986) Antagonists of glutaminergic neurotransmission block retinotectal transmission in goldfish. Brain Res. *398:* 169–174.

Langdon, R. B., and J. A. Freeman (1987) Pharmacology of retinotectal transmission in the goldfish: Effects of nicotinic ligands, strychnine and kynurenic acid. J. Neurosci. *7:* 760–773.

Lipton, S. A., E. Aizenman, and R. H. Loring (1987) Neural nicotinic acetylcholine responses in solitary mammalian retinal ganglion cells. Pfluegers Arch. Eur. J. Physiol. *410:* 37–43.

Masland, R. H., and A. Ames III (1976) Responses to acetylcholine of ganglion cells in an isolated mammalian retina. J. Neurophysiol. *39:* 1220–1235.

Masland, R. H., and C. J. Livingstone (1976) Effect of stimulation with light on synthesis and release of acetylcholine by an isolated mammalian retina. J. Neurophysiol. *39:* 1210–1219.

Masland, R. H., and J. W. Mills (1979) Autoradiographic identification of acetylcholine in the rabbit retina. J. Cell Biol. *83:* 159–178.

Masland, R. H., J. W. Mills, and C. Cassidy (1984) The functions of acetylcholine in the rabbit retina. Proc. R. Soc. London *223:* 121–139.

Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green (1984) Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promotor. Nucleic Acids Res. *12:* 7035–7056.

Millar, T. J., and I. G. Morgan (1987) Cholinergic amacrine cells in the rabbit retina synapse onto other cholinergic amacrine cells. Neurosci. Lett. *74:* 281–285.

Neal, M. J. (1976) Acetylcholine as a retinal transmitter substance. In *Transmitters in the Visual Process,* S. L. Bonting, ed., Chap. 6, pp. 127–143, Pergamon, Oxford.

Needleman, S. B., and C. D. Wunsch (1970) A general method applicable to the search for similarities in the amino acid sequence of two proteins. J. Mol. Biol. *48:* 443–453.

Nef, P., C. Oneyser, C. Alliod, S. Couturier, and M. Ballivet (1988) Genes expressed in the brain define three distinct neuronal nicotinic acetylcholine receptors. EMBO J. *7:* 595–601.

Negishi, K., S. Kato, T. Teranishi, and M. Laufer (1978) An electrophysiological study on the cholinergic system in the carp retina. Brain Res. *148:* 85–93.

Noda, M., H. Takahashi, T. Tanabe, M. Toyosato, Y. Furutani, T. Hirose, M. Asai, S. Inayama, T. Miyata, and S. Numa (1982) Primary stucture of α-subunit precursor of Torpedo californica acetylcholine receptor deduced from cDNA sequence. Nature *299:* 793–797.

Northcutt, G. (1983) Evolution of the optic tectum in ray-finned fishes. In *Fish Neurobiology, Vol. 2: Higher Brain Areas and Functions,* R. E. Davis and R. G. Northcutt, eds., p. 16, University of Michigan Press, Ann Arbor.

Oswald, R. E., and J. A. Freeman (1980) Optic nerve transmitters in lower vertebrates. Life Sci. *27:* 527–533.

Patrick, J., and W. B. Stallcup (1977) Immunological distinction between acetylcholine receptor and the α-bungarotoxin-binding component on sympathetic neurons. Proc. Natl. Acad. Sci. USA *74:* 4689–4692.

Perlman, D., and H. O. Halvorson (1983) A putative signal peptidase recognition site and sequence in eucaryotic and procaryotic signal peptides. J. Mol. Biol. *167:* 391–409.

Proudfoot, N. J., and G. G. Brownlee (1976) 3′ Non-coding region sequences in eucaryotic messenger RNA. Nature *263:* 211–214.

Ricciuti, A., and E. R. Gruberg (1985) Nucleus isthmi provides most tectal choline acetyltransferase in the frog *Rana pipiens.* Brain Res. *341:* 399–402.

Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg (1977) La-

beling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase 1. J. Mol. Biol. *113:* 237–251.

Ross, C. D., and D. A. Godfrey (1986) Effect of enucleation of choline acetyltransferase activity in layers of goldfish optic tectum. Brain Res. *373:* 49–56.

Ross, C. D., and D. B. McDougal, Jr. (1976) The distribution of choline acetyltransferase activity in vertebrate retina. J. Neurochem. *26:* 521–526.

Sanger, F., S. Nicklen, and A. R. Coulson (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA *74:* 5463–5467.

Sargent, P. B., S. H. Pike, D. B. Nadel, and J. M. Lindstrom (1989) Nicotinic acetylcholine receptor-like molecules in the retina, retinotectal pathway, and optic tectum of the frog. J. Neurosci. *9:* 565–573.

Schmidt, J. T., and J. A. Freeman (1980) Electrophysiological evidence that retinotectal synaptic transmission in the goldfish is nicotinic cholinergic. Brain Res. *187:* 129–142.

Schwartz, I. R., and D. Bok (1979) Electron microscopic localization of [^{125}I] α-bungarotoxin binding sites in the outer plexiform layer of the goldfish retina. J. Neurocytol. *8:* 53–66.

Schwartz, M., D. Axelrod, E. L. Feldman, and B. W. Agranoff (1980) Histological localization of binding sites of α-bungarotoxin and of antibodies specific to acetylcholine receptor in goldfish optic nerve and tectum. Brain Res. *194:* 171–180.

Tumosa, N., and W. K. Stell (1986) Choline acetyltransferase immunoreactivity suggests that ganglion cells in the goldfish retina are not cholinergic. J. Comp. Neurol. *244:* 267–275.

Tumosa, N., W. K. Stell, C. D. Johnson, and M. L. Epstein (1986) Putative cholinergic interneurons in the optic tectum of the goldfish. Brain Res. *310:* 365–369.

Vogel, Z., and M. Nirenberg (1976) Localization of acetylcholine receptors during synaptogenesis in retina. Proc. Natl. Acad. Sci. USA *73:* 1806–1810.

von Heijne, G. (1983) Patterns of amino acids near signal-sequence cleavage sites. Eur. J. Biochem. *133:* 17–21.

Woolf, N. J., F. Eckenstein, and L. L. Butcher (1983) Cholinergic projections from the basal forebrain to the frontal cortex: A combined fluorescent tracer and immunohistochemical analysis in the rat. Neurosci. Lett. *40:* 93–98.

Yazulla, S., and J. Schmidt (1976) Radioautographic localization of ^{125}I α-bungarotoxin binding sites in the retinas of goldfish and turtle. Vision Res. *16:* 878–880.

Zucker, C., and S. Yazulla (1982) Localization of synaptic and nonsynaptic nicotinic-acetylcholine receptors in the goldfish retina. J. Comp. Neurol. *204:* 188–195.