Identification of a Novel Nicotinic Acetylcholine Receptor Structural Subunit Expressed in Goldfish Retina

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Abstract. A new nonalpha (nα) member of the nicotinic acetylcholine receptor (nAChR) gene family designated GFnα-2 has been identified in goldfish retina by cDNA cloning. This cDNA clone encodes a protein with structural features common to all nAChR subunits sequenced to date; however, unlike all known α-subunits of the receptor, it lacks the cysteine residues believed to be involved in acetylcholine binding. Northern blot analysis shows multiple transcripts hybridizing to the GFnα-2 cDNA in goldfish retina but undetectable levels of hybridizable RNA in brain, muscle, or liver. S1 nuclease protection experiments indicate that multiple mRNAs are expressed in retina with regions identical or very similar to the GFnα-2 sequence. In situ hybridization shows that the gene encoding GFnα-2 is expressed predominantly in the ganglion cell layer of the retina.

RECENTLY a family of genes encoding nicotinic acetylcholine receptors (nAChRs)[1] expressed at central and peripheral nervous system synapses has been identified by cDNA cloning (22). The number of different members of the nAChR gene family is not known, nor is the functional significance of the different receptor types yet apparent. Understanding the role of nAChRs in the vertebrate central nervous system (CNS) requires the study of a well-defined neural preparation. The vertebrate retina provides such a system since (*a*) it offers a discrete well-defined, and relatively simple laminar anatomy; (*b*) it possesses nicotinic cholinergic synapses, and significant progress has been made in understanding their role in visual processing (1–3, 17, 34, 36, 37); and (*c*) it is accessible to experimental manipulation both in vivo and in vitro.

In contrast to its mammalian counterpart, the optic nerve of the goldfish demonstrates a striking example of CNS regeneration after nerve crush or axotomy (23, 53, 54). The optic nerve is comprised of the axonal processes of the retinal ganglion cells, and it appears that these cells express the nAChR (20, 27, 43, 51). Since regeneration of the optic nerve is marked by changes in gene expression and increased metabolism in retinal ganglion cells (12, 25, 40, 41), regeneration might be expected to modulate expression of nAChR genes. For these various reasons, studies were initiated on nAChRs in the goldfish retina and brain. An additional attraction to the goldfish visual system is the completely crossed optic tracts, providing an experimental and control optic tectum in the same animal after unilateral optic nerve crush (53, 54).

Besides functional nAChRs, retinal cells of most vertebrates also express significant levels of α-bungarotoxin-binding molecules (6, 55, 59, 60). In muscle, α-bungarotoxin binds to the α-subunit of the nAChR (44). In the mammalian nervous system α-bungarotoxin binding is no longer accepted as evidence for the presence of neural nAChRs, since toxin-binding molecules are often found to be distinct from neural nicotinic receptors (13, 46). Nevertheless, in lower vertebrates α-bungarotoxin has been found to bind to a functional neural nAChR synthesized by retinal ganglion cells (19, 27). Interestingly, toxin binding colocalizes with ^{3}H-nicotine binding in the inner plexiform layer of fish, pigeon, and turtle retina (59, 60). However, in goldfish and other nonmammalian vertebrates, one also finds high levels of toxin binding in the outer plexiform layer, a locus at which no nicotine binding is seen (59, 60). Recently two different nicotinic-type binding sites have been isolated from goldfish brain, one of which also binds α-bungarotoxin (26). It is believed that some of these α-bungarotoxin binding-type receptors are synthesized by the retinal ganglion cells and transported to the tectum (19, 27, 51). However, the relationship of these molecules to each other and to other neural nAChRs is not presently known.

To determine the number of different nAChRs expressed in the retina, and to study the expression of the genes encoding these proteins, we have isolated cDNAs corresponding to nAChRs expressed in goldfish retina. Here we detail the identification of a new neural nAChR subunit clone, GFnα-2, isolated from a goldfish retina cDNA library.

Materials and Methods

Isolation of cDNA Clones

Common goldfish (*Carassius auratus*) were used in these studies. Goldfish

1. *Abbreviations used in this paper*: CNS, central nervous system; nAChR, nicotinic acetylcholine receptor; nα, non-alpha.

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                                                                  Met Thr Leu   -26
5'GT TTT TTT TAT TTA TTT TAG CTG GTG TTA CGC AAT ATC AAA CTT TCT CTC AAA ATG ACT CTG   -76

 Ala Val Ile Gly Leu Phe Thr Leu Phe Thr Ser Ile Ile Ala Ile Thr Pro Ala Arg Glu Phe    -5
 GCA GTC ATT GGT CTT TTT ACC CTG TTT ACT AGC ATC ATC GCC ATC ACG CCT GCT AGA GAG TTT   -13
                         1
 Val Ser Leu Ala Glu Arg Glu Asp Ala Leu Leu Arg Glu Leu Phe Gln Gly Tyr Gln Arg Trp    17
 GTA TCT CTT GCA GAA AGA GAA GAT GCT CTT CTT AGG GAG TTG TTT CAG GGG TAC CAG CGC TGG    51

 Val Arg Pro Val Gln His Ala Asn His Ser Val Lys Val Arg Phe Gly Leu Lys Ile Ser Gln    38
 GTC AGG CCG GTT CAG CAT GCA AAC CAC TCT GTG AAG GTC CGC TTT GGA TTG AAG ATA TCT CAG   114

 Leu Val Asp Val Asp Glu Lys Asn Gln Leu Met Thr Thr Asn Val Trp Leu Trp Gln Glu Trp    59
 CTA GTG GAT GTG GAT GAG AAA AAC CAA CTC ATG ACA ACT AAT GTG TGG CTA TGG CAG GAA TGG   177

 Leu Asp Tyr Lys Leu Arg Trp Asn Pro Glu Asn Tyr Gly Gly Ile Thr Ser Ile Arg Val Pro    80
 CTG GAT TAT AAA CTG CGA TGG AAC CCT GAG AAC TAT GGT GGC ATC ACC TCT ATA AGG GTC CCG   240

 Ser Glu Ser Ile Trp Leu Pro Asp Ile Val Leu Tyr Glu Asn Ala Asp Gly Arg Phe Glu Gly   101
 TCA GAG AGC ATC TGG CTC CCA GAC ATT GTT TTA TAT GAA AAT GCT GAT GGA CGT TTT GAA GGC   303

 Ser Leu Met Thr Lys Ala Ile Val Arg Tyr Asn Gly Met Ile Thr Trp Thr Pro Pro Ala Ser   122
 TCA CTT ATG ACC AAA GCC ATC GTG CGG TAC AAC GGC ATG ATC ACA TGG ACG CCT CCT GCC AGC   366

 Tyr Lys Ser Ala Cys Thr Met Asp Val Thr Phe Phe Pro Phe Asp Arg Gln Asn Cys Ser Met   143
 TAC AAG TCC GCC TGC ACC ATG GAT GTG ACC TTT TTC CCA TTC GAT CGG CAG AAC TGC TCT ATG   429

 Lys Phe Gly Ser Trp Thr Tyr Asp Gly Asn Met Val Lys Leu Val Leu Ile Asn Gln Gln Val   164
 AAG TTT GGC TCT TGG ACA TAT GAT GGA AAC ATG GTC AAA CTA GTC CTC ATC AAC CAG CAG GTG   492

 Asp Arg Ser Asp Phe Phe Asp Asn Gly Glu Trp Glu Ile Leu Ser Ala Thr Gly Val Lys Gly   185
 GAC CGA AGC GAC TTC TTT GAT AAC GGC GAG TGG GAG ATT CTC AGC GCC ACT GGT GTC AAA GGC   555

 Ser Arg Gln Asp Ser His Leu Ser Tyr Pro Tyr Ile Thr Tyr Ser Phe Ile Leu Lys Arg Leu   206
 AGT CGG CAA GAC AGC CAC CTC TCC TAC CCC TAC ATC ACG TAC TCG TTC ATC TTG AAA CGT CTT   618

 Pro Leu Phe Tyr Thr Leu Phe Leu Ile Ile Pro Cys Leu Gly Leu Ser Phe Leu Thr Val Leu   227
 CCT CTC TTC TAC ACA CTC TTC CTC ATT ATT CCC TGC CTG GGC TTG TCC TTC CTA ACA GTG CTG   681

 Val Phe Tyr Leu Pro Ser Asp Glu Gly Glu Lys Val Ser Leu Ser Thr Ser Val Leu Val Ser   248
 GTC TTC TAC CTG CCC TCT GAT GAA GGG GAA AAA GTC TCT CTC TCC ACC TCC GTC CTG GTG TCC   744

 Leu Thr Val Phe Leu Leu Val Ile Glu Glu Ile Ile Pro Ser Ser Ser Lys Val Ile Pro Leu   269
 CTC ACT GTG TTC CTT CTC GTC ATC GAG GAG ATC ATC CCT TCT TCT TCC AAG GTT ATC CCG CTG   807

 Ile Gly Glu Tyr Leu Leu Phe Ile Met Ile Phe Val Thr Leu Ser Ile Ile Val Thr Ile Phe   290
 ATT GGA GAG TAC CTG CTT TTT ATC ATG ATT TTT GTC ACT CTC TCC ATC ATC GTG ACC ATT TTT   870

 Val Ile Asn Val His His Arg Ser Ser Ala Thr Tyr His Pro Met Ser Pro Trp Val Arg Ser   311
 GTG ATC AAC GTC CAC CAC CGC TCT TCA GCT ACT TAC CAT CCC ATG TCT CCG TGG GTG CGT TCG   933

 Leu Phe Leu Gln Arg Leu Pro His Leu Leu Cys Met Arg Gly Asn Thr Asp Arg Tyr His Tyr   332
 CTG TTT CTC CAG CGG CTG CCT CAT TTG CTC TGC ATG AGG GGA AAC ACA GAC CGC TAT CAT TAC   996

 Pro Glu Leu Glu Pro His Ser Pro Asp Leu Lys Pro Arg Asn Lys Lys Gly Pro Pro Gly Pro   353
 CCA GAG CTG GAG CCT CAC AGC CCT GAC CTT AAG CCC AGG AAC AAG AAA GGG CCA CCT GGC CCT  1059

 Glu Gly Glu Gly Gln Ala Leu Ile Asn Leu Leu Glu Gln Ala Thr Asn Ser Val Arg Tyr Ile   374
 GAA GGA GAA GGT CAA GCT CTG ATT AAT CTG CTG GAG CAG GCT ACC AAC TCG GTT CGC TAC ATC  1122

 Ser Arg His Ile Lys Lys Glu His Phe Ile Arg Glu Val Val Gln Asp Trp Lys Phe Val Ala   395
 TCA CGC CAC ATT AAG AAG GAG CAT TTT ATT AGG GAG GTA GTT CAG GAC TGG AAG TTT GTG GCT  1185

 Gln Val Leu Asp Arg Ile Phe Leu Trp Thr Phe Leu Thr Val Ser Val Leu Gly Thr Ile Leu   416
 CAG GTG TTG GAC AGG ATT TTT CTC TGG ACC TTC CTC ACA GTC TCT GTG CTC GGC ACC ATC CTC  1248

 Ile Phe Thr Pro Ala Leu Lys Met Phe Leu Arg Thr Pro Pro Pro Pro Ser Pro ***          434
 ATC TTT ACA CCT GCC CTC AAG ATG TTC CTG CGC ACA CCT CCA CCT CCT TCT CCG TGA CAC TTG  1311

 AGT CAT TCC ATT CAA ATA CAT CAC ATC GCA AAA GGA AAT TTT ATC TAC CAC TAA CAT TAC AGA  1374

 CAG TGC ACA TTA CTA TGT AAA TGT AAT TTG TTC GTT CAC TGC ATT AAA GAC TCA AAT GTA TGA  1437

 TTA ACA ATG TAA GAT GAC CAT GTT AAA CAA ATG TGA AGT TAG CAG AGT AGC ATT ATT TGT CCT  1500

 TAG TGA TAC GTT TAA AAC TTT TGT TTG TAG ATT ATT TAA TTA TTA ATT CTC AAA ATT GTA TGT  1563

 ACA CTC AAC ATA AAA AAC TCA CAA AAA AGA AAG AAA AAA AAA ACT TAT TTT GAC TTA TGC AAG  1626

 AAA AAA TTA AAT CTC CCA AGA ATT AGA AAA AAA AAA AAA...3'                              1665
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Figure 1. Nucleotide and deduced amino acid sequence of cDNA clone GFnα-2. Nucleotides are numbered in the 5′ to 3′ direction starting with the first nucleotide in the codon corresponding to 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.

were anesthetized and optic nerves were crushed as described (18). 7 and 10 d after optic nerve crush, goldfish were dark adapted and retinas were removed and stored at −80°C. RNA was isolated from retinas using the guanidine thiocyanate procedure (52). Poly(A)$^+$ RNA was selected by chromatography over an oligo(dT)-cellulose column (4). Poly(A)$^+$ RNA (2.5 μg) from 7- and 10-d regenerating retinas were pooled and used to prepare the cDNA library. RNA was reverse transcribed into cDNA with AMV reverse transcriptase (30), linkered with Eco RI phosphorylated linkers, and then cloned into the Eco RI site of bacteriophage lambda gt10 (30).

5×10^5 recombinant phage were screened with a radiolabeled mixed α-subunit probe consisting of nick-translated (48) cDNAs encoding the α-subunits of the Torpedo electric organ AChR (45), kindly provided by Dr. Norman Davidson (California Institute of Technology), the rat muscle AChR, and the rat neural AChR α-4 subunit (22). Filters were hybridized in 5× SSPE (0.75 M NaCl, 57 mM NaH_2PO_4, 5 mM EDTA [pH 7.4]), 5× Denhardt's, 100 μg/ml denatured herring sperm DNA, 0.1% SDS at 65°C, and washed in 5× SSC (0.75 M NaCl, 0.075 M Na citrate [pH 7.0]), 0.1% SDS at 65°C (35).

This screening resulted in the isolation of six cDNAs that encode three different goldfish neural nAChR subunits. One of these (GFnα-2) is characterized in the results.

Northern Blot Analysis and S1 Nuclease Protection Experiments

For Northern blots, poly(A)$^+$ RNA was isolated as described above, denatured at 65°C, and then subjected to electrophoresis in 2.2-M formaldehyde–1.2% agarose gels (47). The RNA was then transferred to a Gene Screen Plus membrane. Prehybridization and hybridization conditions were 5× SSPE (0.75 M NaCl, 57 mM Na_2HPO_4, 5 mM EDTA [pH 7.4]), 1%

SDS, 10% dextran sulfate, and 50% formamide at 45°C. After hybridization, the blot was washed in 1× SSC (0.15 M NaCl, 0.015 M Na citrate [pH 7.0]), 1% SDS at 55°C, and exposed to x-ray film with an intensifying screen at −70°C. High stringency, posthybridization washes were carried out in 0.1% SDS, 0.1× SSC at 55°C.

S1 nuclease digestions of heteroduplexes formed between poly(A)$^+$ RNA and M13 subclones of GFnα-2 were carried out as previously described (21). For the deletion mapping experiment, retinal poly(A)$^+$ RNA was hybridized with M13 subclones containing complementary GFnα-2 cDNA corresponding to either the full-length cDNA or clones containing deletions at their 5′ end (see Fig. 4). These latter clones contained GFnα-2 3′ DNA extending 5′ for 1,415, 1,157, 934, or 713 bases. Those hybrids surviving S1 nuclease digestion were analyzed by electrophoresis through a 1.2% agarose–formaldehyde gel, transferred to Gene Screen Plus, and detected by hybridization to nick-translated radiolabeled GFnα-2 cDNA.

In Situ Hybridization

In situ hybridization was performed as previously described (14). In brief, goldfish retinas were removed and fixed in ice cold 4% paraformaldehyde, PBS (pH 7.4). After 2–4 h, retinas were transferred to ice cold 30% sucrose in PBS overnight. Retinas were then transferred to room temperature O.C.T. (Tissue Tek) for 15 min. and then frozen at −70°C in O.C.T. 20-μm-thick sections were cut and mounted on polylysine-coated slides. Before hybridization sections were treated for 15 min with 5 μg/ml proteinase K, and acetylated with acetic anhydride. Sections were hybridized with single-stranded, ^{35}S-labeled RNA probes. These probes were prepared by run-off transcription of linearized pGEM-4 vectors containing the GFnα-2 cDNA insert lacking its poly(A) tail. The cDNA poly(A) tail was removed by restricting with Dra-1 at nucleotide 1650. Antisense RNA probes were transcribed with T7 RNA polymerase and sense probes with SP6 RNA polymerase (38). Hybridization was performed at 55°C for 12–16 h in 50% deionized formamide, 10% dextran sulfate, 300 mM NaCl, 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 0.1 mg/ml RNase-free *Escherichia coli* tRNA (Boehringer Mannheim Diagnostics, Inc., Houston, TX) and 10 mM DTT. Posthybridization treatments included a wash in 50% formamide, 2× SSC at 65°C and an RNase digestion to reduce background (18). Slides were then dehydrated, dipped in Nuclear Track Emulsion NTB 2, air dried, and exposed 1–2 wk at 4°C. After developing, sections were stained with hematoxylin-eosin B.

DNA Sequence Determination

DNA sequencing was performed using the dideoxynucleotide chain termination method (49). cDNAs were subcloned into M13 bacteriophage vectors mp18 and mp19. Unidirectional deletions were generated using T4 DNA polymerase (15).

Results

Isolation of a cDNA Encoding a Putative nAChR Structural Subunit Expressed in Goldfish Retina

The vertebrate retina expresses both nAChRs and α-bungarotoxin–binding molecules (1–3, 6, 34, 36, 37, 55, 60). The relationship of these molecules to each other and to other CNS nAChRs is not known nor is their function understood. To address these issues and to develop probes to study nAChR gene expression in the retina, it is necessary to isolate DNA clones encoding these molecules. To identify retinal nAChR genes, cDNA libraries were prepared in lambda gt10 using poly(A)$^+$ RNA isolated from goldfish retinas. Libraries were screened with a radiolabeled mixed receptor probe (see Materials and Methods). Screening 5×10^5 recombinants resulted in the purification of six different lambda recombinant clones. Based on restriction enzyme digestion profiles and partial DNA sequence analysis, these six clones were determined to represent three classes of molecules. One class, represented by GFnα-2, appeared to represent a new nAChR subunit and was characterized further.

Clone GFnα-2 is 1,850 nucleotides long, with an open reading frame of 1,387 bp (Fig. 1). This open reading frame begins with an initiator ATG at position −84 and ends with a stop codon, TGA, at position 1302. The 5′ end of the clone contains 53 bases of untranslated sequence. The 3′ end of the clone contains a poly(A) tail ~50 nucleotides long (not shown in Fig. 1) that is preceded by two polyadenylation signal sequences at residues 1572 and 1631. Based on a comparison of the deduced amino acid sequence of GFnα-2 with the other nAChR subunit sequences and taking into account the sequence patterns around the signal sequence cleavage site (56), we have designated the NH_2 terminus of the mature GFnα-2 protein to be a glutamic acid residue. This results in the identification of the first 28 residues as comprising a leader peptide.

Comparison of the deduced amino acid sequence of clone GFnα-2 with the muscle and neural nAChR subunit sequences shows that this clone encodes a protein that is a member of the nAChR gene family (Fig. 2). The GFnα-2 protein is most similar to the rat neural α4 subunit (22) (~55% sequence identity). It exhibits ~50% sequence identity with the rat neural α2 (57) and α3 (8) proteins and ~45% identity with both the mouse muscle α1 (7) and rat neural β2 proteins (16). The Drosophila ARD protein (28) contains ~40% sequence identity with the goldfish GFnα-2 subunit.

Analysis of the GFnα-2 protein sequence indicates it contains many structural features common to all neural nAChR subunits sequenced to date (5). These include (*a*) four hydrophobic putative transmembrane domains; (*b*) an extracellular β-loop structure before the first hydrophobic domain formed by cysteines 127 and 140, with a turn induced by the conserved proline at position 135; (*c*) two potential N-linked glycosylation sites at positions 25 and 140; and (*d*) a hydrophilic domain situated between the third and fourth hydrophobic domains.

The protein encoded by GFnα-2 lacks cysteines 191 and 192, which are found in all nAChR α-subunits sequenced to date. These cysteines are believed to be close to the agonist binding domain (32). In this respect GFnα-2 is more similar to the non-α (nα)-subunits of muscle and neural nAChRs. Based on the absence of these adjacent cysteine residues in GFnα-2 and the conservation of the many structural domains between this protein and all other neural nAChR subunits, we propose that GFnα-2 represents a nonagonist-binding subunit of a novel class of neural nAChRs.

GFnα-2 cDNA Identifies Two Different Retinal RNAs

The distribution of GFnα-2 gene expression in the goldfish was analyzed by Northern blots (Fig. 3). Poly(A)$^+$ RNA was isolated from liver, skeletal muscle, brain, and retina. Poly(A)$^+$ RNA (5 μg) was size fractionated on denaturing agarose gels and transferred to a Gene Screen Plus membrane. The blot was probed with radiolabeled GFnα-2 cDNA and washed at high stringency (0.1× SSC, 0.1% SDS 55°C) before exposing to x-ray film. This experiment showed GFnα-2 cDNA to hybridize to at least two different RNAs in retinal tissue corresponding to ~2.4 and 1.8 kb. No detectable signal was observed in lanes containing RNA isolated from brain, muscle, or liver.

S1 nuclease protection experiments were used to determine if any of the RNAs seen hybridizing with the GFnα-2

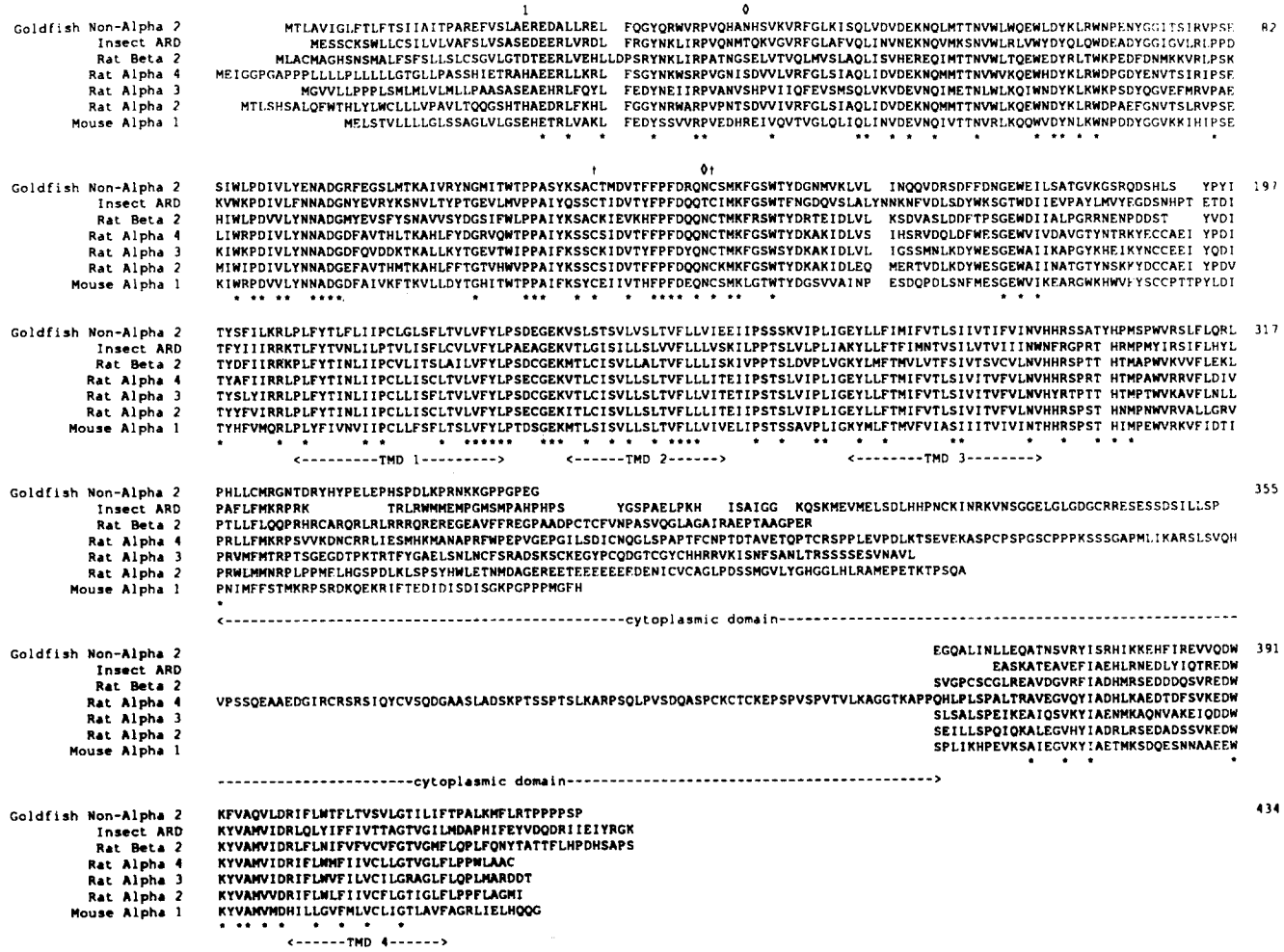

Figure 2. Comparison of deduced amino acid sequences for acetylcholine receptor α and nα subunits. Shown are α and nα subunit sequences from mouse muscle (α1) (7), rat neuronal α-subunits (α2, α3, and α4) (8, 22, 57), rat neuronal nα-subunit (β2) (16), Drosophila nα-subunit (ARD) (28), and the goldfish nα-subunit (GFnα-2). Amino acids conserved in all seven subunits are indicated by an asterisk. The putative transmembrane and cytoplasmic domains are indicated below the aligned sequences. Diamonds indicate potential glycosylation sites and daggers indicate conserved cysteine residues.

probe on Northern blots contained regions that were identical or very similar in sequence to the GFnα-2 DNA. For these experiments GFnα-2 was subcloned into the single-stranded phage M13mp19 in the antisense orientation. This DNA was next hybridized with retinal poly(A)$^+$ RNA. S1 nuclease was then added to digest any single-stranded nucleic acids not forming heteroduplexes. Those molecules surviving digestion were fractionated on a denaturing agarose gel, transferred to a Gene Screen Plus membrane, and visualized by hybridizing to a nick-translated GFnα-2 cDNA insert. This experiment resulted in the identification of two different RNAs protected by the full-length (~1,850 nucleotides) GFnα-2 probe (Fig. 4). The larger (1.8-kb) band represents complete protection of the RNA by the probe while the smaller protected band represents a second RNA that has ~850 nucleotides in common with the GFnα-2 clone.

To map along the GFnα-2 sequence where this second RNA shares homology, we generated a series of GFnα-2 subclones that contained deletions from their 5′ ends (Fig. 4 *A*). These subclones were then used in S1 nuclease protection experiments and the size of the protected bands was determined on agarose gels (Fig. 4 *B*). One predicts that, as the 5′ end of GFnα-2 is deleted, the band corresponding to the fully protected RNA will decrease in size, corresponding to that of the deleted probe. However, the second, partially protected, RNA only decreases in size when hybridized with a subclone containing a 5′ deletion that overlapped the region of identity between GFnα-2 and this RNA. When this experiment is carried out, one finds the second RNA to decrease in size only when an M13 subclone is used that contains a 5′ deletion extending beyond nucleotide 435 (M13 probe 3′ 1415 in Fig. 4). Therefore, this second RNA shares a sequence encoding the first three hydrophobic domains and part of the cytoplasmic domain of GFnα-2. Controls, in which RNA was omitted from the hybridization reaction, showed no signal (Fig. 4, −).

GFnα-2 Is Expressed in the Ganglion Cell Layer of the Retina

The distribution of cells in the retina expressing the GFnα-2

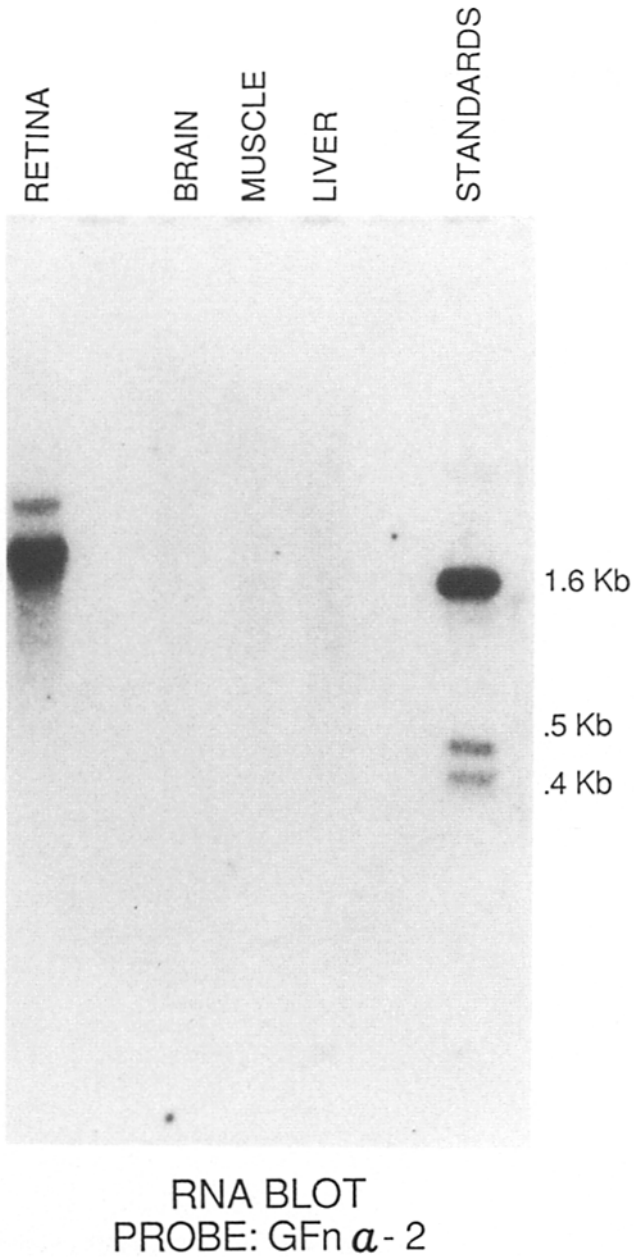

Figure 3. Northern blot analysis. 5 μg of poly(A)$^+$ RNA, isolated from goldfish retina, brain, muscle, and liver was size fractionated on a 2.2-M formaldehyde–1.2% agarose gel and transferred to Gene Screen Plus membrane. The blot was probed with ^{32}P-labeled GFnα-2 cDNA, and washed at high stringency.

gene was determined by in situ hybridization. Retinal sections were hybridized with single-stranded ^{35}S-labeled antisense RNA probes. Probes were prepared by run-off transcription of linearized pGEM-4 vectors containing GFnα-2 cDNA insert lacking its poly(A)$^+$ tail. Antisense probes consistently hybridized to RNA in cells of the ganglion cell layer (Fig. 5). The distribution of cells in this layer that showed positive hybridization to the probe indicate that not every cell of the ganglion cell layer expresses the GFnα-2 gene (Fig. 5 *D*). Controls for nonspecific hybridization included sense strand probes (Fig. 5 *C*) and pretreating sections with RNase before hybridization with the antisense probe (data not shown). These treatments resulted in only background hybridization. Sections were also examined before staining to verify that grains resulting from hybridization were not obscured by the histological stains used to visualize individual cells in the ganglion cell layer.

Discussion

The diversity of nAChRs expressed in the CNS is larger than might be anticipated from pharmacological approaches. Determining the function of the proteins encoded by these genes is a challenging problem. We have decided to study nAChR expression in the retina because its laminar organization and relatively small number of cell types make it amenable to experimental approaches.

To date, three different nAChR α-subunit-like genes expressed in the CNS have been identified and are referred to as α2, α3, and α4 (8, 22, 57). A single α-subunit encoding gene (α1) has been found to be expressed in skeletal muscle (7, 31). Four other genes (β, γ, δ, and ε), expressed in skeletal muscle, encode structural subunits of the nAChR (9, 10, 33, 39). In mammalian neural tissue a single nα nAChR gene has been identified. This gene product can substitute for the β-subunit of the muscle nAChR to form a functional receptor, and so it is referred to as β2 (16).

Based on protein purification, amino acid sequence analysis, and cDNA cloning, it appears that at least one neural nAChR is likely to be a tetramer composed of two identical α-subunits and two identical β2-subunits (58). Expression studies have shown that a functional nAChR can be formed when β2 is combined with any of the neural α-subunits (16). In addition, it has been found that the β2 gene is expressed in the same areas of the brain as the α-subunit genes (16). These results have led to the proposal that different nAChRs are expressed by combining the β-subunit with different α-subunits (16). It is not yet clear whether other nα-subunits exist that combine with α-subunits to form neural nAChRs. We report here the identification of a second nα-subunit (GFnα-2) isolated from a goldfish retinal cDNA library. Like β2, this clone is classified as a nα-like subunit because it lacks adjacent cysteine residues 192 and 193 that are near the agonist binding site found in all α-subunits (32).

DNA sequence analysis shows GFnα-2 encodes a protein with structural features common to all nAChR subunits. These features include (*a*) a hydrophilic amino-terminal domain; (*b*) four hydrophobic putative transmembrane domains; (*c*) a hydrophilic putative cytoplasmic domain situated between the third and fourth hydrophobic domain; (*d*) conserved cysteines corresponding to residues 128 and 142 of the Torpedo α-subunit (45); and (*e*) two potential N-linked glycosylation sites at positions 25 and 140. The conservation of these features between the various nAChR subunits, and between the members of the family of ligand-gated ion channel genes, implies that they play an important role in receptor function (5).

The deduced amino acid sequence of clone GFnα-2 is more similar to the rat α4 sequence (55%) than any other known nAChR subunit (Fig. 2). It is of interest that GFnα-2 encodes a protein that has more sequence identity with the α-subunits (45–55%) than the nα-subunits (40–45%) of the muscle and neural receptors. Based on these amino acid se-

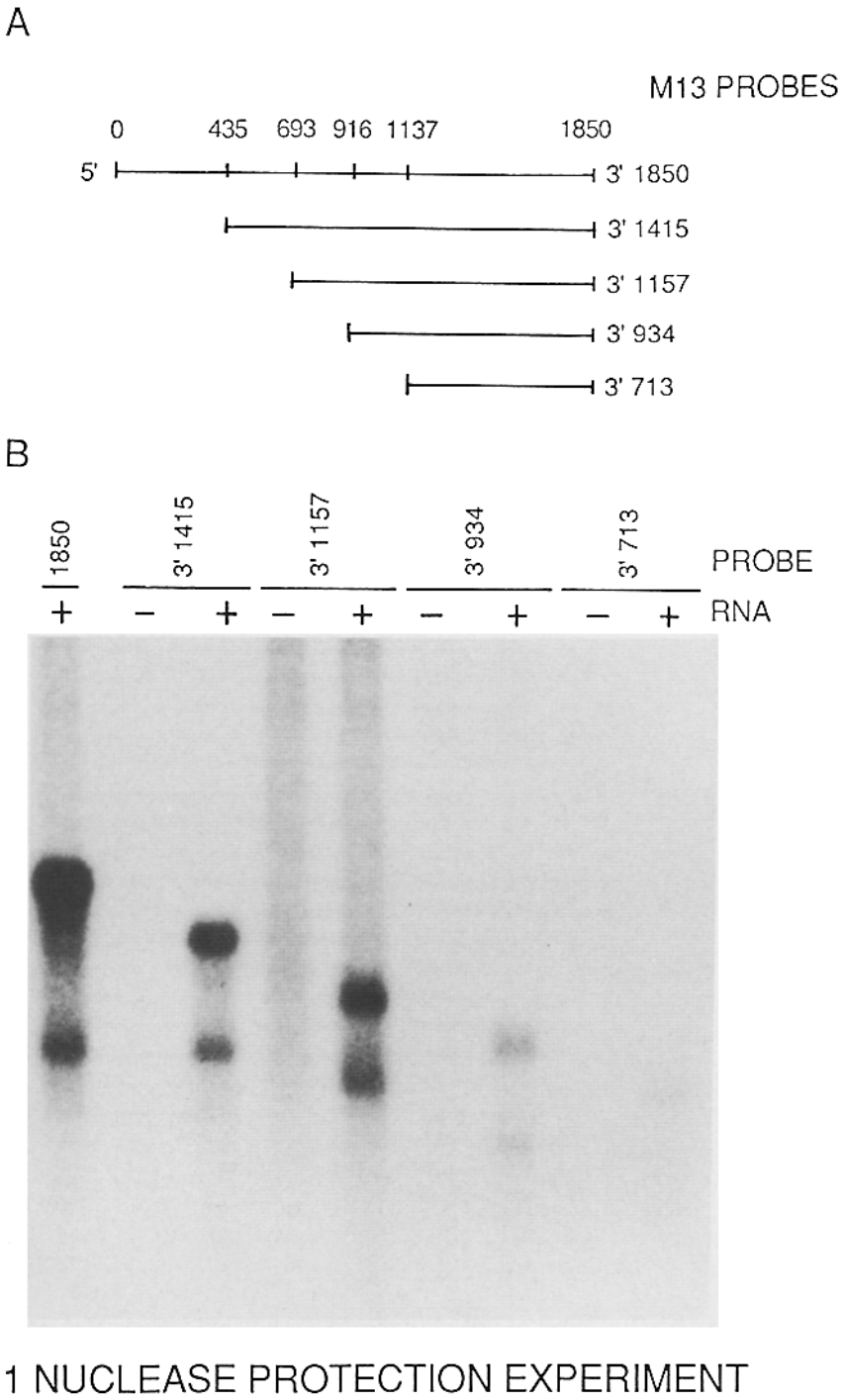

Figure 4. S1 nuclease protection experiment. (*A*) Line diagram of M13 subclones used to hybridize with retinal RNA. The full-length clone is 1,850 bases long. Deletions of the 5′ end were generated, and these M13 subclones were named according to the number of nucleotides remaining at the 3′ end. (*B*) Gel profile of S1 nuclease-protected fragments generated by S1 nuclease digestion of heteroduplexes formed between poly(A)$^+$ RNA isolated from retinal tissue and the M13 probes shown in *A*. + lanes contain RNA and − lanes are controls lacking RNA. The two bands in lane *1850+* are ~1,850 and 850 bases long.

quence comparisons, it is clear that GFnα-2 represents a new member of the nAChR gene family. We believe this gene family is similar to that found to exist in chick and rat since at least two other members of this gene family are also expressed in fish. First, the α1 gene encoding the agonist-binding subunit of the muscle nAChR has been shown to be expressed in fish, chick, and mammals (7, 18, 45). Second, we have isolated another goldfish cDNA whose amino-terminal DNA sequence has been determined and exhibits ~80% DNA sequence identity with the rat and chick α3 DNA sequences. We believe this molecule represents the goldfish homologue of the rat and chick α3 gene. Thus, we predict that birds and mammals also express the gene corresponding to GFnα-2 in goldfish.

The level of GFnα-2 gene expression in goldfish was investigated by Northern blot analysis (Fig. 3). A significant level of expression is found in retina, but it is not detectable in brain, muscle, or liver tissue. This is in contrast to the level of expression of the neural β2 gene, whose RNA is expressed at higher levels in chick brain than in chick retinal tissue (50). Thus, the GFnα-2 gene product may contribute to a retinal-specific nAChR.

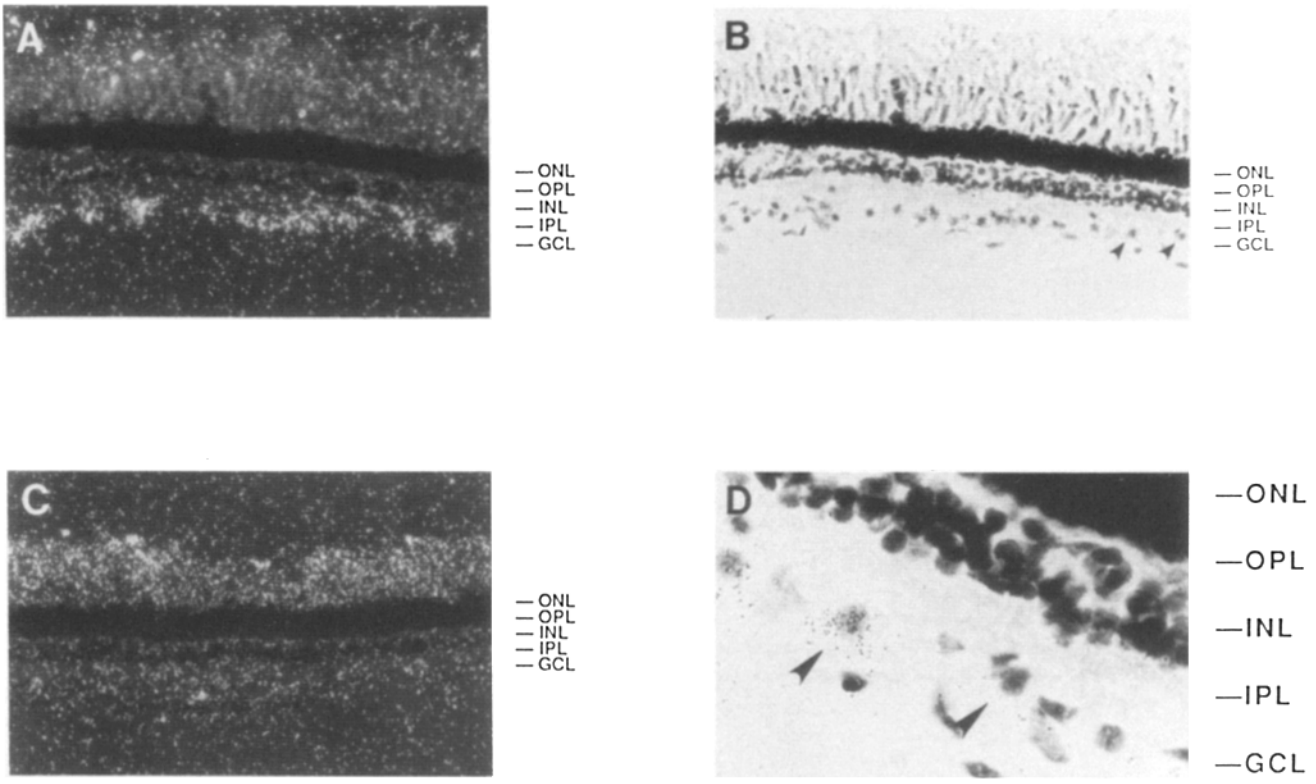

Figure 5. In situ hybridization of GFnα-2 to sections of goldfish retina. Goldfish retinal sections were hybridized with ^{35}S-labeled RNA corresponding to GFnα-2 cDNA in the antisense (*A*, *B*, and *D*) or sense (*C*) orientation. Sections were stained with hematoxylin and eosin B. *A* and *C* are dark-field illumination (20×) and *B* and *D* are bright field illumination (20× and 100× oil, respectively). *B* is a bright-field picture of the same section in *A*, and *D* is a magnified region of the ganglion cell layer shown in *B* (*arrowheads*). *ONL*, outer nuclear layer; *OPL*, outer plexiform layer; *INL*, inner nuclear layer; *IPL*, inner plexiform layer; and *GCL*, ganglion cell layer.

The Northern blot data indicates that multiple retinal RNAs hybridize to the GFnα-2 DNA. This heterogeneity was confirmed by S1 nuclease protection experiments, showing that a second retinal RNA shares ~850 bases of sequence identity with GFnα-2 (Fig. 4). The completely protected RNA corresponds to the GFnα-2 gene product while the second partially protected RNA may represent a second gene product or alternative splicing of a single GFnα-2 primary transcript. Assuming a similar structure for the GFnα-2 gene as the other neural nAChR genes, the region of identity between these two different RNAs closely corresponds to exon 5, which spans the first three hydrophobic domains and part of the cytoplasmic domain (42). This implies that, in addition to GFnα-2, another nAChR subunit is expressed in the retina differing from GFnα-2 at its amino and carboxy termini.

In situ hybridization was used to determine which cells in the goldfish retina express the GFnα-2 gene. Since GFnα-2 encodes a protein with structural features common to all nAChR subunits sequenced to date, we expected to find this gene expressed in those cells known to synthesize nAChRs. Consistent with this prediction, we find GFnα-2 gene expression in the ganglion cell layer of the retina (Fig. 5).

Retinal ganglion cells likely express functional nAChRs postsynaptically to cholinergic amacrine cells (1–3, 20, 24, 34, 36, 37). In addition, goldfish retinal ganglion cells may express nAChRs presynaptically at the retinotectal synapse (19, 27, 51). At least some of these presynaptic receptors bind α-bungarotoxin (19, 27, 51). These receptors are not likely to be the same gene products as those found postsynaptically in the retina since the latter receptors are not blocked by α-bungarotoxin (43). The GFnα-2 gene may encode the structural subunit for one or both of these molecules. Immunocytochemical localization of the GFnα-2 protein will be necessary to distinguish between these possibilities.

S1 nuclease protection experiments showed that in addition to the GFnα-2 gene, retinal cells express a second gene encoding a protein very similar to the GFnα-2 gene product (Fig. 4). Since the in situ hybridization was performed with a hydrolyzed full-length antisense GFnα-2 RNA probe, the signal obtained is the result of hybridization to both the fully and partially protected RNAs seen in the S1 protection experiment. It appears that both of these transcripts are expressed in the ganglion cell layer. In situ hybridization using probes corresponding to nonhomologous regions of these two RNAs will permit more detailed analysis of their cellular distribution.

In situ hybridization experiments have consistently shown a subset of cells in the ganglion cell layer to express the GFnα-2 gene. While the reasons for this are not known, there are, however, a few possibilities. The types of cells making up the ganglion cell layer may be reflected in gene expression. Not all cells in the vertebrate retinal ganglion cell layer are retinal ganglion cells; amacrine cells, displaced from the inner nuclear layer, and glial-like cells can be found there as well (11, 24, 29). These additional cells would be

expected to contribute to nonuniformities in the in situ hybridization pattern. In contrast to many other species, the percentage of displaced amacrine cells in the ganglion cell layer of the goldfish retina is quite small (29) and so is not expected to contribute significantly to the overall hybridization pattern of GFnα-2. Based upon the nicotinic nature of the retinal ganglion cell response characterized in goldfish (20, 43) and other species (1–3, 36, 37) we believe that GFnα-2 is expressed by retinal ganglion cells, and not by the glial-like cells present in the ganglion cell layer. Ganglion cells can be classified electrophysiologically as ON, OFF, or ON-OFF cell types as determined by how they discharge in response to a focal light stimulus (17). These electrophysiological properties can be correlated with the ganglion cells' response to exogenously applied acetylcholine and nicotinic agonists and antagonists (1–3, 20, 36, 37, 43). These different responses may reflect differential expression of nAChR genes. Further experiments will test this hypothesis.

In conclusion, we have presented the isolation and characterization of a cDNA clone encoding a putative nAChR subunit, expressed in the retina. GFnα-2 represents the second nα-like subunit expressed in the vertebrate CNS identified to date. This clone is proposed to encode a structural subunit of a retinal nAChR based on DNA sequence and predicted protein homology with other nAChR subunits. Consistent with this proposal, we find the gene encoding GFnα-2 expressed in the retinal ganglion cell layer, a region known to express nAChRs. Evidence is also provided for the existence of a second transcript expressed in the retinal ganglion cell layer that contains regions very similar to GFnα-2. This RNA may represent yet another nAChR subunit gene or be the result of alternative processing of the GFnα-2 primary transcript.

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