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

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Abstract. A new nonalpha (no0 member of the nicotinic acetylcholine receptor (nAChR) gene family designated GFno0-2 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 GFno0-2 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 GFno0-2 sequence. In situ hybridization shows that the gene encoding GFno0-2 is expressed predominantly in the ganglion cell layer of the retina.

Recently a family of genes encoding nicotinic acetylcholine receptors (nAChRs) 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 3H-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, GFno0-2, isolated from a goldfish retina cDNA library.

Materials and Methods

Isolation of cDNA Clones

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

**Figure 1. Nucleotide and deduced amino acid sequence of cDNA clone GFna-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 to base 1 are designated by negative numbers and include residues encoding the putative signal peptide and 5' untranslated sequence. Polyadenylation signal sequences are underlined in the 3' untranslated region.

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 GeneScreen Plus membrane. Prehybridization and hybridization conditions were 5x 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 (GFna-2) is characterized in the results.

**Northern Blot Analysis and 31 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 5x SSC (0.75 M NaCl, 0.075 M NaHPO₄, 5 mM EDTA [pH 7.4]), 1% Denhardt's, 100 μg/ml denatured herring sperm DNA, 0.1% SDS at 65°C, and washed in 5x SSC (0.75 M NaCl, 0.075 M Na citrate [pH 7.0]), 0.1% SDS at 65°C (35).
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.

2. Nuclease digestions of heteroduplexes formed between poly(A+) RNA and M13 clones of GFna-2 were carried out as previously described (21). For the deletion mapping experiment, retinal poly(A+) RNA was hybridized with M13 subclones containing complementary GFna-2 cDNA corresponding to either the full-length cDNA or clones containing deletions at their 5′ end (see Fig. 4). These latter clones contained GFna-2 3′ DNA extending 5′ for 1,415, 1,157, 934, or 713 bases. Those hybrids surviving SI 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 GFna-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 OCT (Tissue Tek) for 15 min. and then frozen at ~70°C in OCT. 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, 35S-labeled RNA probes. These probes were prepared by run-off transcription of linearized pGEM-4 vectors containing the GFna-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 (15). 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 E. 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 dideoxyxynucleotide chain termination method (49). cDNAs were subcloned into M13 bacteriophage vectors mpl8 and mpl9. Unidirectional deletions were generated using T4 DNA polymerase (15). These cysteines are believed to be close to the agonist binding domain (32). In this respect GFna-2 is more similar to the non-α (να)-subunits of muscle and neural nACHRs. Based on the absence of these adjacent cysteine residues in GFna-2 and the conservation of the many structural domains between this protein and all other neural nACHR subunits, we propose that GFna-2 represents a nonagonist-binding subunit of a novel class of neural nACHRs.

GFna-2 cDNA Identifies Two Different Retinal RNAs

The distribution of GFna-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 GFna-2 cDNA and washed at high stringency (0.1× SSC, 0.1% SDS 55°C) before exposing to x-ray film. This experiment showed GFna-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.

SI nuclease protection experiments were used to determine if any of the RNAs seen hybridizing with the GFna-2
Figure 2. Comparison of deduced amino acid sequences for acetylcholine receptor α and α subunits. Shown are α and α subunit sequences from mouse muscle (α1) (7), rat neuronal α-subunits (α2, α3, and α4) (8, 22, 57), rat neuronal α-subunit (α2) (16), Drosophila α-subunit (AR4) (28), and the goldfish α-subunit (GFαα-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 GFαα-2 DNA. For these experiments GFαα-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 protection experiments, in which RNA shares homology, we generated a series of GFαα-2 subclones that contained deletions from their 5′ ends (Fig. 4A). These subclones were then used in S1 nuclease protection experiments and the size of the protected bands was determined on agarose gels (Fig. 4B). One predicts that, as the 5′ end of GFαα-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 GFαα-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 the cytoplasmic domain of GFαα-2. Controls, in which RNA was omitted from the hybridization reaction, decrease in size only when hybridized with a subclone containing a 5′ deletion that overlapped the region of identity between GFαα-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 the cytoplasmic domain of GFαα-2. Controls, in which RNA was omitted from the hybridization reaction, showed no signal (Fig. 4, -).

**GFαα-2 Is Expressed in the Ganglion Cell Layer of the Retina**

The distribution of cells in the retina expressing the GFαα-2 probe on Northern blots contained regions that were identical or very similar in sequence to the GFαα-2 DNA. For these experiments GFαα-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 protection experiments, in which RNA shares homology, we generated a series of GFαα-2 subclones that contained deletions from their 5′ ends (Fig. 4A). These subclones were then used in S1 nuclease protection experiments and the size of the protected bands was determined on agarose gels (Fig. 4B). One predicts that, as the 5′ end of GFαα-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 GFαα-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 the cytoplasmic domain of GFαα-2. Controls, in which RNA was omitted from the hybridization reaction, showed no signal (Fig. 4, -).
tions 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 αα 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 αα-subunits exist that combine with αα-subunits to form neural nAChRs. We report here the identification of a second αα-subunit (GFαα-2) isolated from a goldfish retinal cDNA library. Like β2, this clone is classified as an αα-like subunit because it lacks adjacent cysteine residues 192 and 193 that are near the agonist binding site found in all αα-subunits (32).

The deduced amino acid sequence of clone GFαα-2 is more similar to the rat α4 sequence (55%) than any other known nAChR subunit (2). It is of interest that GFαα-2 encodes a protein that has more sequence identity with the α-subunits (45–55%) than the αα-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.

Sequence 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 GFα-2 to sections of goldfish retina. Goldfish retinal sections were hybridized with 35S-labeled RNA corresponding to GFα-2 cDNA in the antisense (A, B, and D) or sense (C) orientation. Sections were stained with hematoxylin and eosin. A and C are dark-field illumination (20x) and B and D are bright field illumination (20x and 100x 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 GFα-2 DNA. This heterogeneity was confirmed by S1 nuclease protection experiments, showing that a second retinal RNA shares ~850 bases of sequence identity with GFα-2 (Fig. 4). The completely protected RNA corresponds to the GFα-2 gene product while the second partially protected RNA may represent a second gene product or alternative splicing of a single GFα-2 primary transcript. Assuming a similar structure for the GFα-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 GFα-2, another nAChR subunit is expressed in the retina differing from GFα-2 at its amino and carboxy termini.

In situ hybridization was used to determine which cells in the goldfish retina express the GFα-2 gene. Since GFα-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 GFα-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 GFα-2 gene may encode the structural subunit for one or both of these molecules. Immunocytochemical localization of the GFα-2 protein will be necessary to distinguish between these possibilities.

S1 nuclease protection experiments showed that in addition to the GFα-2 gene, retinal cells express a second gene encoding a protein very similar to the GFα-2 gene product (Fig. 4). Since the in situ hybridization was performed with a hydrolyzed full-length antisense GFα-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 GFα-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 GFncr-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 GFncr-2 is expressed by retinal ganglion cells, and not by the glia-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, 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. GFncr-2 represents the second α-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 GFncr-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 GFncr-2. This RNA may represent yet another nAChR subunit gene or be the result of alternative processing of the GFncr-2 primary transcript.

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