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The Goldfish as a Model for Studying Neuroestrogen Synthesis, Localization, and Action in the Brain and Visual System

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Organizational and activational effects of estrogen (E) in the central nervous system (CNS) are exerted directly by circulating E and indirectly after aromatization of circulating androgen to E in the brain itself. Understanding an environmental chemical's ability to disrupt E-dependent neural processes, therefore, requires attention to both pathways. Because aromatase (Aro) is highly expressed in teleost brain, when compared to mammals and other vertebrates, fish are technically advantageous for localization and regulation studies and may also provide a model in which the functional consequences of brain-derived (neuro-E) synthesis are examined. Recently, Aro was immunolocalized in cell bodies and fiber projections of second- and third-order neurons of the goldfish retina and in central visual processing areas. Authentic Aro enzyme activity was verified biochemically, suggesting a hitherto unrecognized role of sex steroids in the visual system. Initial studies show that in vivo treatment with aromatizable androgen or E increases calmodulin synthesis and calmodulin protein in retina and also affects retinal protein and DNA. Whether there are related changes in the processing of visual information that is essential for seasonal reproduction or in the generative and regenerative capacity of the goldfish visual system requires further investigation. — Environ Health Perspect 103(Suppl 7):51–57 (1995)

Key words: estrogen, aromatase, brain, retina, goldfish

Introduction

Many steroidal and nonsteroidal chemicals mimic natural estrogens (E) in receptor binding and other biological properties. Epidemiological studies in humans, together with observation of wild-caught animals, suggest that estrogenlike chemicals present in the environment or administered as contraceptive or therapeutic drugs [xenoestrogens (XEs)] might be responsible for perturbations of sexual differentiation, sexual maturation, and adult reproductive processes in both sexes.

Although most attention has focused on structural or functional abnormalities of the external genitalia, gonads, or reproductive tract, the brain is both a target and a source of E. Understanding an environmental chemical's potential to affect brain development and physiology, therefore, requires attention to the dual role of natural E in the central nervous system (CNS). As a prerequisite to the study of XEs, in this paper we review available information and present data that suggests that the visual system of the goldfish may be an advantageous model.

The Brain as an Estrogen Target

E has functionally important neural actions in all vertebrate species studied to date (1–3). During certain critical periods of early development, exposure to E affects permanent change in organizational processes, which are revealed subsequently as male–female differences in brain structure or function. At the onset of sexual maturation and in sexually mature animals, a transient increase in E serves as a feedback signal that positively or negatively regulates the brain–pituitary–gonadal axis and activates certain sex and sex-related behaviors. By virtue of an ability to take up and retain 1H-estradiol (E2), neurons with classical nuclear estrogen receptors (ER) were first mapped by autoradiographic methods and found to be concentrated mainly, but not exclusively, in forebrain areas known to control reproduction and sex behavior, i.e., preoptic area (POA), hypothalamus (HTH), and other limbic components. These are phylogenetically “ancient” brain regions and have similar distributions of ER-positive cells from fish to mammals. Studies using ER antibody or oligonucleotide probes, as well as ER-binding analysis, electrophysiological techniques, E-implants, lesioning, and double-labeling techniques, have given us a comprehensive understanding of E target cells within the CNS, their exact location, ontogeny, phenotypic characteristics, and physiology. As in other E targets, ER are ligand-activated transcription factors that bind to specific DNA sequences in the promoter region of target genes and regulate transcription; therefore, analysis of downstream elements of genetic response cascades initiated by E in specific cells and circuits is one strategy for identifying molecular markers of XE exposure.

The presence of nuclear ER does not exclude the possibility that E can operate via additional, nongenomic mechanisms at the same or different sites in the CNS. For example, 2- or 4-hydroxylated metabolites of E2 or estrone (E1) (termed catecholestrogens), which are formed in brain, have an A-ring that resembles catecholamines structurally. Some catecholestrogens are able to bind to nuclear ER and thereby mimic or block effects of natural E and also bind to catecholamine receptors, inhibit tyrosine
hydroxylase, and serve as substrates of hydroxy-O-methyltransferase action (4–6). Steroid binding sites have been described on outer cell membranes where they are thought to mediate short latency electrophysiological responses (7,8), and additional evidence indicates that some steroids interact with nonsteroid receptor classes (9,10). Accordingly, ER binding per se is not a reliable predictor of an environmental chemical’s ability to disrupt E-dependent neural processes. The functional relevance of E actions not involving nuclear ER and biomarkers of alternative mechanisms may be revealed in the future using ER-knockout mice (11,12).

The Brain as a Site of Estrogen Synthesis

Although E is generally regarded as a hormone derived from glandular secretions, it can be synthesized in the brain itself from circulating androgen and, in this context, operates as a parahormone. The rate-limiting step in E synthesis is regulated by a cytochrome P450 enzyme complex termed aromatase (Aro), which is encoded by a member of the cytochrome P450 gene superfamily (CYP19)(13). Using 3H-androgen to measure E products in a catalytic assay, Naftolin and Ryan (14) made the first definitive identification of Aro in the HTH and limbic system of the newborn rodent and human fetus, and this was subsequently extended to other mammalian and nonmammalian species (1–3). Many different lines of evidence show that aromatization in situ is functionally important for the full expression of neural androgen actions. Furthermore, effector pathways that are initiated by brain-derived (neuro)-E are appear to be spatially or temporally uncoupled from those initiated by gonad-derived (circular) E. For example, an aromatizable androgen (testosterone, T) secreted by the neonatal rodent testis normally feminizes the brain of males, but E2 that is present in the circulation of both males and females is apparently without effect. By contrast, the nonsteroidal estrogen diethylstilbestrol (DES) is highly potent. One explanation is that plasma E2, but not DES, is bound to circulating a-fetoprotein, which reduces effective concentrations at functionally important brain sites. Without testing for access to and actions in the CNS, it cannot be assumed that information developed by screening for XE effects on peripheral tissues can be extrapolated directly to brain. Even chemicals without ER binding or biological activities can interfere with E-sensitive brain targets if they are aromatizable, they block aromatization of endogenous substrates, they affect catalytic activity, or they otherwise alter Aro gene expression.

The Goldfish Brain Model

Compared to mammals and other vertebrates, teleost fish have exceptionally high levels of brain Aro activity, e.g., 1300 fmole versus 1 to 5 fmole/mg protein/hr in the hypothalamic/preoptic area (HPOA) of goldfish versus rat, mouse, rabbit or hamster, respectively (3). In addition to forebrain areas generally regarded as part of the reproductive brain, enzyme activity is readily detected throughout the midbrain, hindbrain, and spinal cord (15,16). Also, evidence that Aro activity is present in the pituitary was first obtained in fish (17) and was subsequently confirmed in mammalian and avian pituitary (18,19). High expression appears to be limited to neuroendocrine tissues, however, because ovarian Aro activity is less than 1/10 that of brain, and other tissues are Aro-negative. Northern analysis using a goldfish brain-specific Aro cDNA confirms results of enzyme assays by showing high transcript levels in neural but not ovarian tissues, indicating that overexpression of enzyme is due to a pretranslational mechanism [Gelinas et al., unpublished data (20)].

After perfusion of the teleost telencephalon in situ with 3H-androgen, recovery of 3H-E region-by-region exactly corresponds to enzyme activity (HTH/P0A > remaining forebrain > mid/hindbrain)(21). Thus, high rates of aromatization operate under simulated in vivo conditions and account for high, region-specific levels of accumulating neuroE. Because ER binding affinity (Kd = 10^-8 M) and activity in goldfish brain are in the range reported for mammals and other vertebrates (3), E synthesis in excess of that required for maximal ER occupancy may be necessary to reach concentrations needed to evoke nongenomic actions. Analysis of spent perfusate also shows that the teleost brain may contribute quantitatively important amounts of free and conjugated E to the general circulation and to the aquatic environment via the gills and hepatic and renal pathways (22). Free and conjugated steroids have been implicated as teleostean pheromones.

An association between neuroE synthesis and reproduction in goldfish is supported by studies showing that plasma levels of aromatizable androgen, brain Aro activity, and Aro mRNA all increase > 5-fold at the onset of seasonal reproductive activity and return to baseline in reproductively inactive fish (23). Seasonal changes in Aro activity or Aro mRNA in forebrain can be mimicked by treating fish with aromatizable androgen or E, but non-aromatizable androgen [5α-dihydrotestosterone, (DHT)] is ineffective [Gelinas et al., unpublished data; (24)]. We interpret this to mean that neuroE is part of an autoregulatory positive feedback loop that effectively amplifies neuroE actions by directly or indirectly increasing Aro gene transcription (24–26).

Estrogen Synthesis and Action in the Goldfish Visual System

Using a polyclonal rabbit antibody raised against partially purified human placental Aro (Gelinas and Callard, unpublished data), the majority of immunoreactive (ir) Aro neurons were located in reproductive control centers of the goldfish telencephalon and diencephalon, a distribution that corresponded to highest catalytic activity (Figure 1). Additionally, irAro neurons and fibers were present in the retina and included a subset of horizontal cells (HC), bipolar cells (BP), amacrine cells (AC), and...
ganglion cells (GC), which often appeared to contact each other via irAro-positive processes; but photoreceptors were never labeled (27). Some GC projections to the brain via the optic nerve and optic tract were positive for irAro, as were small neurons of the stratum periventriculare (SPV) and fibers of two other strata of the optic tectum [Gelinias and Callard, unpublished data; (27)]. Although the optic tectum is the primary target of retinal projections in goldfish, irAro-labeled cells are located in other areas known to receive retinal or tectal projections (e.g., suprachiasmatic nucleus; area Dc of the telencephalon) and in premotor areas of the hindbrain that integrate visual, auditory, and vibrational information (e.g., cerebellum, vestibular neurons, and Mauthner cells) (Figure 1).

Aro enzyme was verified in the retina and tectum by catalysis of 3H-androgen to authentic E using a standard homogenate assay and primary cell cultures. Recently, ER-specific mRNA was reported in trout retina (28), and an autoradiographic study of toadfish brain revealed labeled cells in the optic tectum after peripheral 3H-T and 3H-E injection (29,30). Taken together, these data suggest a role for neuroE in regulating gene transcription but do not rule out nongenomic actions in the telost visual system. Most telosts are highly visual and rely on retina-mediated photic and other visual input for seasonal gonadal recrudescence, diurnal timing of ovulation, mate identification, and spawning (31). A striking feature of the telost CNS, including the visual system, is an ability to continue to grow throughout life and to regenerate structurally and functionally after damage (32). Here we report initial attempts to determine the role of neuroE in these processes as a preliminary to the study of xenobiotic actions.

Methods

Animals

Adult male and female goldfish (12–14 cm total body length) were obtained from Grassyfork Fisheries (Martinsville, IN) and maintained under lighting conditions of 12:12 LD at a water temperature of 17°C. Gonadectomy was carried out after immersion anesthesia using 0.06% 2-phenoxyethanol, and steroids were administered by implanting silastic capsules (1.5 mm dia×10 mm long; Dow Corning Corp., Midland, MI) into the abdominal cavity as previously described (24). 35S-Methionine (> 1000 μCi/mmole; Amersham, Arlington Heights, IL) and 3H-thymidine (90Ci/mmole; New England Nuclear, Boston, MA) were administered to anesthetized fish by intraocular injection and, after sacrificing the fish by decapitation, optic tecta and retinae were collected. These procedures have been described previously (33).

Protein and DNA Analysis

For analysis of 3H-thymidine-labeled DNA, tissues were homogenized in 0.1 M phosphate buffer (K+, pH 7.2). To solubilize tissues for protein analysis, tissue aliquots were extracted according to O'Farrell (34). Total amino acid or thymidine uptake were measured by β-liquid scintillation spectrosopy in sample aliquots diluted in Optifluor (New England Nuclear, Boston, MA). To measure radioactivity in the macromolecular fraction (protein or DNA), additional aliquots were treated with ice cold trichloroacetic acid (TCA), final concentration 12%, precipitates washed with 80% ice cold acetone, and the pellets solubilized in 0.1 N NaOH. Protein was measured using bovine serum albumin (BSA) as standard (35), and DNA was measured using salmon sperm DNA (Boehringer Mannheim, Indianapolis, IN) as standard (36). Values were expressed as dpm per μg protein or DNA. Aliquots of radiolabeled proteins (equivalent radioactivity) from different treatment groups were separated on two-dimensional (2D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (34). Spots were visualized after treatment of gels with EnHance and exposure to Hyperfilm-β-max (both from New England Nuclear) for 3 weeks. Additional aliquots of solubilized protein (equivalent protein) from different treatment groups were separated on one-dimensional (1D) SDS-PAGE gradient gels (5–20%) and transferred to nitrocellulose membrane for Western analysis using a mouse monoclonal anticalmodulin antibody (Sigma Chemical Company, St. Louis, MO) and the ECL System (New England Nuclear) to visualize bound antibody. Gels were scanned using an LKB Ultrascan XL Enhanced Laser Densitometer in combination with a GelScan XLTM Software Program (Pharmacia, Piscataway, NY).

Results

Experiment 1

To determine effects of aromatizable androgen [testosterone, (T)] on overall retinal protein synthesis and transport, reproductively inactive fish (November and December) were gonadectomized and received either blank or T-filled silastic implants. After 3 weeks, 35S-methionine (80 μCi, 8 μl) was injected intraocularly, and 48 hr later retinae and tecta were processed for radiolabeled amino acid uptake and protein incorporation. As shown in Table 1, T treatment in vivo decreased amino acid uptake and protein incorporation by 50% in the injected retina; this was accompanied by a 50% reduction of transported radiolabeled protein to the contralateral tectum. Radioactivity was at background levels in retinae from the uninjected eye and ipsilateral tectum.

Experiment 2

To assess effects of E, aromatizable and nonaromatizable androgen on specific metabolically labeled retinal proteins, reproductively inactive fish (Dec) were implanted with capsules containing no steroid, T, E2, or 5α-dihydrotestosterone (DHT). After 1 week, 35S-methionine (70 μCi, 7 μl) was administered by intraocular injection and fish sacrificed 48 hr later. Total amino acid uptake and incorporation in the retinae and tecta of fish with intact gonads were unaffected by 1 week of steroid treatment (n = 4–6/treatment; data not shown). After 2D SDS-PAGE most protein spots were similar in electrophoretic mobility and labeling intensity; however, relative amounts of a low-molecular-weight acidic protein (arrows) increased 10- and 5-fold after treatment with E2 and T, respectively, and decreased about 50% after DHT

Table 1. Effect of sex steroids on 35S-methionine uptake and incorporation in the retina and optic tectum (experiment 1).

| Treatment      | Uptake (Total cpm) | Incorporation (Total cpm) |
|----------------|-------------------|-------------------------|
|                | cpm/μg protein    | cpm/μg protein          |
| Retina         |                   |                         |
| Gonadectomy    | 3,223,050         | 3,688                   |
| Tectum         | 35,500            | 42                      |
| E2 + T         | 1,926,030         | 2,338                   |
| Retina         | 3,688             | 42                      |
| Tectum         | 1,926,030         | 2,338                   |

Values are means of two experiments. *Testosterone.
Anatomic weight was seen. Ameasured wet weight was also decreased per an experiment. In the untreated controls (n = 6/treatment; one experiment). After 2 weeks, 3H-thymidine (4 µCi; 4 µl) was injected intraocularly, and incorporation into retinal DNA was measured 24 hr later. As shown in Table 2, there were no significant differences when untreated controls and fish receiving an empty capsule were compared. No T effect was seen on the rate of DNA synthesis, as measured by 3H-thymidine incorporation per unit DNA or per unit protein; however, T significantly increased retinal wet weight 30% (p < 0.01) and decreased DNA content per retina by the same percentage (p < 0.001), when compared to controls. These changes were not attributable to an artifact of dissection, because the DNA/protein ratio of the assayed samples also decreased markedly after T (p < 0.001).

Discussion

Anatomic evidence alone indicates that sex steroids have a heretofore unrecognized role in the goldfish visual system. Additional evidence is provided by differential analysis of synthesized proteins, which suggests that neuroE formed in situ from circulating T is associated with elevated calmodulin expression in the retina. Many Ca** activated enzymes, including adenylyl cyclase and those involved in synthesis and release of neurotransmitters at the synapse, are dependent on the Ca** binding protein calmodulin (38). In turn, changes in neuronal activity alter calmodulin activity and distribution, with consequences for subsequent Ca** dependent events. Adrenalectomy decreases and corticosteroids restore calmodulin III-specific mRNA in cerebral cortex and hippocampus of rats and induce corresponding changes in adenylyl cyclase activity (39). Corticosterone also enhances calmodulin binding when added directly to highly purified synaptic membranes (40); low doses of E are reported to increase neuronal nitric oxide synthase (NNOS) activity in rat cerebellar cytosol by a Ca** calmodulin-dependent mechanism that is independent of ER (41). High concentrations of Aro activity in synaptosomal subfractions of brain (42) and the presence of iAro in neuronal extensions and fiber layers of the visual system [Gelinas and Callard, unpublished data (27)] provide an anatomic basis for postulating that neuroE has actions in both presynaptic and postsympathetic neurons by a two-step mechanism involving immediate nongenomic actions on calmodulin activity and subsequent changes in calmodulin protein.

Calmodulin protein is known to double during the cell cycle, independent of changes in activation state due to Ca** (38), and is associated with the assembly and disassembly of microtubules and other structural proteins, which are established markers of neuronal growth, process extension, and synaptogenesis. Although results of experiments reported here suggest an

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**Table 2. Effects of aromatizable androgen on retinal protein, DNA, and 3H-thymidine incorporation (experiment 3).**

| Treatment          | WW*, mg/retina | DNA, µg/retina | Protein, µg/retina | DNA/protein ratio | 3H-Thymidine incorporation |
|--------------------|----------------|----------------|--------------------|-------------------|---------------------------|
|                    |                |                |                    |                   | DNA, dpm/µg | Protein, dpm/µg |
| None               | 57 ± 1         | 95 ± 12        | 560 ± 98           | 0.17 ± 0.03       | 919 ± 308 | 141 ± 46 |
| Empty capsule      | 53 ± 1         | 63 ± 6         | 478 ± 68           | 0.19 ± 0.02       | 860 ± 196 | 147 ± 25 |
| Testosterone       | 69 ± 1*        | 57 ± 5**       | 702 ± 105          | 0.09 ± 0.01**     | 1131 ± 278 | 100 ± 28 |

*WW, wet weight. Values are means ± SEM, six animals per treatment group. *p < 0.01; **p < 0.001
organizational effect of steroids in the visual system, data are difficult to interpret without further experimentation. The most striking changes after treatment with aromatizable androgen for 1 week are an increase in retinal weight and a decrease in retinal DNA content and DNA/protein ratio, although the rate of DNA synthesis was unaffected. This suggests a steroid-induced increase in cell death together with an expansion of surviving neurons. Paradoxically, 3 weeks of T treatment after gonadectomy decreases overall protein synthesis. Steroids are known to account for cycles of neuronal growth and regression in avian song control nuclei (43,44) and in the rodent hypothalamus (45), but in these models, at least in reproductive brain regions, E is a neurotrophic factor. In goldfish, the demands of increased visual activity associated with reproduction might not be compatible with continuous neuronal growth, proliferation, and synaptic reorganization which is characteristic of the retina and tectum in this species. If so, sex steroids may signal a temporary arrest of cell division and remodeling processes. Although it has been reported that tectal reorganization after partial ablation is affected by time of year, day-length and size of fish, the reproductive status of animals used in this study was not defined (46).

There is little direct information indicating that electrophysiological or behavioral-visual attributes change in relation to season or reproductive status; however, in one study, fish were found to have greater sensitivity to the photostimulatory effects of long days as the reproductive season approached (47). It is worth noting here that gonadotropin releasing hormone (GnRH) has a prominent role in the goldfish visual system. Terminals immunoreactive for GnRH are localized in the retina where they envelop all classes of second-order neurons (48,49), which are also ArO-labeled. Many irGnRH fibers are seen in SAC of the optic tectum (48-51), a fibrous layer adjacent to SPV which has numerous irArO-positive cells.

In isolated superfused goldfish retina, GnRH has actions consistent with an excitatory neurotransmitter and changes the spectral sensitivity of GC (48,49). As in mammals, GnRH synthesis, release, and actions on gonadotropin synthesis and secretion are potentiated by E (52-54), and neuroE may have a similar role in the visual system.

Can results in goldfish be generalized to other species? Published micrographs show a few weakly labeled irArO neurons in the rat lateral geniculate nucleus and moderate numbers of labeled cells in the striate (visual) cortex (55,56). In a postmortem study of human retina, several C-21 steroids as well as E were detected by radioimmunoassay (57), which signifies either uptake from the circulation or synthesis. In a separate study, Lanthier and Patwardhan (58) claim that rat retinal tissues are capable of transforming radiolabeled androgen to E, among other steroid transformations, but data were not presented. A few investigators have used the anterior chamber of the eye to study hormonal effects on neural tissues (59), suggesting that steroids taken up from the blood or synthesized in situ are present in vitreal fluid. In a clinical study of 110 women with intraocular metastases, 77% were derived from mammary tumors, a type which is E-dependent in early stages (60).

Compared to glucocorticoid actions in the eye, we know relatively little about the effects of gender, reproduction, and sex steroid status on the visual system. Although the adult mammalian visual system does not continue to grow and regenerate, maternal exposure to exogenous steroids during early embryonic critical periods is a real possibility if we take into account the continued accumulation of natural and synthetic E in the environment and the wide use of anabolic steroids and oral contraceptives by the healthy population. For example, one side effect of oral contraceptives is the blurring of vision (61), and tamoxifen use in breast cancer patients is associated with retinopathy, although the latter may be due to factors other than its estrogenic/antiestrogen activity (62,63). A study of aging in quail reports that light-induced retinal damage, characterized by accumulation of lipofuscin, is virtually female specific (64), and an earlier study described ovarian steroid effects on photoreceptor damage in rats (65). How exposure to synthetic E during aging in postmenopausal women or to decreasing levels of aromatizable androgen in both sexes as a part of normal aging might affect visual processes is unknown.

Because of its accessibility and relative organizational simplicity compared to brain, the neuronal circuitry, neurochemistry, electrophysiology, and development of the goldfish retina and optic tectum has been extensively studied (32,66). Thus, we may anticipate that the teleost visual system will be advantageous for testing effects of EXE and other environmental chemicals on normal visual functions and will lead to recognition of E-controlled processes in the human visual system during critical stages of early development, explain male-female differences in visual functions, allow predictions in steroid-exposed adults or after reproductive aging, and assist in devising strategies to prevent neurodegeneration.

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