ANALYSIS OF THE GOLDFISH (*CARASSIUS AURATUS*) AROMATASE (P450AROM) GENE PROMOTERS BY GREEN FLUORESCENT PROTEIN (GFP) EXPRESSION IN LIVING ZEBRAFISH (*DANIO RERIO*) EMBRYOS

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It is well established that estrogen has permanent, organizational effects on CNS development. Although estrogen is generally regarded as a circulating hormone derived from the gonads, access of estrogen to neural targets is limited by high levels of plasma binding proteins or neural metabolizing enzymes. Thus, developmental effects are dependent on estrogen formed from circulating androgen at sites of action within the brain itself. Aromatization of androgen to estrogen is catalyzed by cytochrome P450 aromatase (P450arom), a product of the *CYP19* gene, and this transformation regulates the quantity of ligand available for binding to estrogen receptors. Teleosts are unique in having exceptionally high brain levels of P450arom when compared to the ovaries of the same fish (300-fold higher) or to the brains of other vertebrates (100- to 1000-fold higher) (Callard, G.V. et al., Gen. Comp. Endocrinol. 43:243-255, 1981). This makes teleosts a good model to study the synthesis and action of estrogen in the brain. We previously reported that the goldfish has two P450arom isoforms which are encoded by separate and unique gene loci, *CYP19A* and *CYP19B* (Tchoudakova, A. and Callard, G.V., Endocrinology 139:2179-2189, 1998). Neural tissues express high levels of P450aromB and much lower levels of P450aromA, whereas P450aromA is the only isoform expressed in the ovary where levels are very low. Recently we isolated and cloned the 5'-flanking regions of the two *CYP19* genes. Sequence analyses showed a low percent identity between the promoter regions of *CYP19A* and *B*, suggesting that they differ with respect to critical cis-acting regulatory elements. Analysis of fish promoters is hampered by the lack of suitable fish cell lines and their resistance to transfection. Given the close taxonomic relationship of goldfish and zebrafish, the goal of the present study was to test the utility of the zebrafish embryo as an in vivo whole animal system for functional analysis of *CYP19* gene transcription.

5'-Flanking regions of the goldfish CYP19 genes comprising 1.5 (*CYP19B*) and 1.0 (*CYP19A1*) or 0.7 kb (*CYP19A3*) were subcloned into the plasmid pEGFP-1 (Clontech), which contains the GFP structual gene as a reporter. Maintenance and breeding of zebrafish were as previously described (Westerfield, M. "The Zebrafish Book", the University of Oregon Press, Eugene, OR, 1993). Embryos at the one- to four-cell stage (up to 1.5 hr postfertilization, pf) were injected with 4.6 nl of supercoiled plasmid DNA (30 or 100 µg/ml 0.1M KCl), and screened for GFP expression under a fluorescent microscope (Olympus IMT2) at intervals up to 48 hr pf. The images were recorded using a confocal microscope (Olympus Fluoview Personal Confocal Microscope System). Six of 43 injected embryos surviving at 30 hr pf exhibited fluorescence in neuron-like cells in the brain, when approximately 100 embryos were injected with a construct containing the *CYP19B* promoter (Fig. 1). At 48 hr pf the signal in forebrain was absent, but labeled cells were now seen in mid- and hindbrain. No expression of GFP was observed in the embryos injected with the *CYP19A1* or *CYP19A3* promoter constructs.

The results demonstrate that expression of *CYP19B* is brain-specific and occurs very early in zebrafish development, implying a functional role for locally synthesized estrogen in early neural development. This finding, and our inability to detect expression of *CYP19A*/GFP constructs, agrees with RT-PCR analysis of staged embryos, which shows P450aromB-specific mRNA at 6 hr pf and delayed expression of P450aromA until 48 hr pf (unpublished data). Further studies are required to define the exact onset and time course of expression and to identify the neuron-like cells which express GFP. We conclude that zebrafish embryos are a relevant system for future studies of brain-specific, developmentally-programmed, and hormone-regulated mechanisms involved in *CYP19B* transcription. The study was supported by a grant from NSF IBN9605053 (GVC) and NIEHS P30 E503828 (MDIBL Center for Membrane Toxicity Studies).

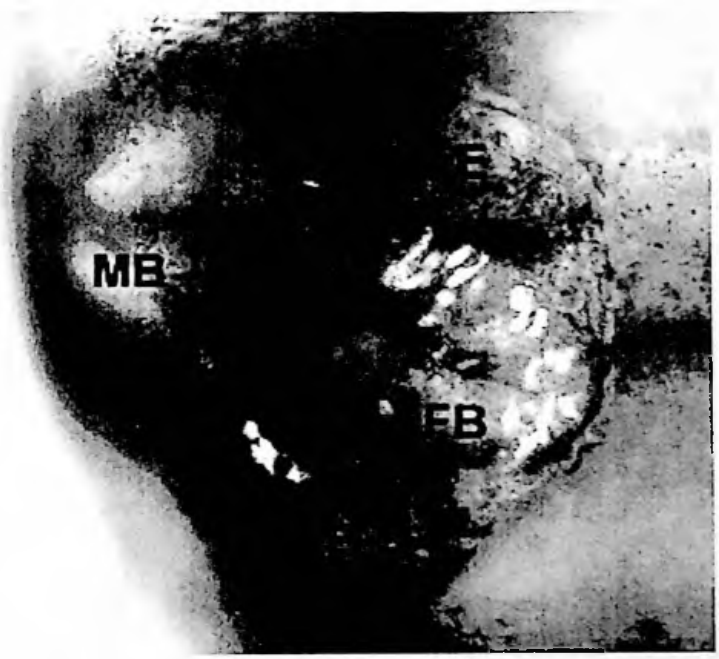

Figure 1. Transient expression of GFP in a zebrafish embryo (30 hr pf) injected with the *CYP19B1*/GFP construct showing neuron-like cells in the brain. FB, forebrain; MB, midbrain; E, eye.