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Studies on the neuroendocrine regulation of puberty and reproductive function

Magliulo-Cepriano, Lucia, Ph.D.

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U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

Studies on the Neuroendocrine Regulation of Puberty and Reproductive Function

by

Lucia Magliulo-Cepriano

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4/15/94
Date

Martin P. Schreibman
Chairman of Examining Committee
Dr. Martin P. Schreibman, Brooklyn College

4/19/94
Date

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Supervising Committee

The City University of New York

Abstract

Studies on the Neuroendocrine Regulation

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Lucia Magliulo-Cepriano

Adviser: Dr. Martin P. Schreibman

Xiphophorus maculatus, a small freshwater teleost commonly known as the platyfish, has served as a valuable model for investigations in to the regulation of sexual maturation and reproductive physiology. This study has elucidated some of the neuroregulatory factors that may impact on gonadotropin releasing hormone (GnRH) neurons and on pituitary functions involved in growth and reproduction, through the immunocytochemical mapping of neuroregulatory factors in the brain and pituitary gland of *Xiphophorus* at significant stages of development from birth to sexual maturation.

The immunocytochemical distribution of FMRF-amide, galanin, neurotensin, neuropeptide Y, dynorphin, dopamine, serotonin, androgen receptors, Gonadotropin (GTH) I, GTH II, salmon GnRH, chicken II GnRH, mammalian GnRH, and lamprey

GnRH in the brain and pituitary gland is detailed in this report. The results gathered have lead us to the following conclusions:

1. We have established that FMRF-amide- and neuropeptide Y-like peptides are located in the nucleus olfactoretinalis (NOR), a brain nuclei believed to be of major importance in mediating events involved in the orchestration of puberty and of reproductive function. We have further established that the NOR is not a homogenous cluster of neurons but rather a heterogenous mixture of morphologically similar but physiologically different neurons that serve different functions.

2. Multiple forms of GTH and GnRH are present in the platyfish and are associated with specific stages of development. Specifically, GTH II and chicken II GnRH appear in later stages of puberty while GTH I , mammalian GnRH, salmon GnRH and lamprey GnRH are present from early developmental stages and throughout sexual maturation. Lamprey GnRH was seen only in the pituitary gland.

3. Neural regulation of pituitary function is accomplished by means of a network of regulatory events. This network includes a number of pituitary cell types and a variety of neuroregulatory factors all of which are capable of interacting with one another under the appropriate conditions.

Our findings on the temporal appearance and distribution of neuroregulatory factors gives us a better understanding of the process of sexual maturation and of the neurophysiology of reproductive function in platyfish.

This dissertation is dedicated to the memory of my mother,

Virginia Criscuolo Magliulo

whose goodness and love

live on

in her children,

Theresa, Agostino, Michael, Gennaro, Angela, Emilia, and Maria Rose.

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List of Abbreviations

ACTH	adrenocorticotropin hormone
AVT	arginine vasotocin
B	brain
BK	Bradykinin
C	cerebellum
ch	chicken
CPD	caudal pars distalis
DA	dopamine
DYN	dynorphin
EV	end vesicle of pineal
GABA	gamma aminobutyric acid
GAL	galanin
GH	growth hormone
GLU	glutamate
GnRH	gonadotropin releasing hormone
GTH	gonadotropin
l	lamprey
m	mammalian
NH	neurohypophysis
NLT	nucleus lateralis tuberis
NOR	nucleus olfactoretinalis
NPO	nucleus preopticus
NPP	nucleus preopticus periventricularis
NPY	neuropeptide Y
NRL	nucleus recessus lateralis
NRP	nucleus recessus posterioris
NT	neurotensin
OL	olfactory bulb
OC	optic chiasm
OT	optic tectum
P	pituitary gland
PI	pars intermedia
PRL	prolactin
PS	pineal stalk
R	rhombencephalon
RPD	rostral pars distalis
s	salmon
SL	somatolactin
SP	substance P
T	telencephalon
TSH	thyrotropin stimulating hormone
VC	valvular cerebellum
VIP	vasoactive intestinal peptide
VT	ventral tegmentum
III	third ventricle

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Introduction

I. Brief history of the field of neuroendocrine regulation of pituitary function.

The concept of the pituitary as the "master gland" came into being in the early 1930's with the discovery of pituitary hormones and a growing understanding of their functions. While it was accepted by some that this minuscule endocrine organ orchestrated and maintained endocrine physiology, hypothalamic control of pituitary function had actually been suggested as far back as 1921 with studies conducted by Bailey and Bremer in which they lesioned the base of the brain of dogs. This resulted in a reproduction of symptoms associated with adiposigenital dystrophy, a syndrome involving obesity, retarded gonadal development and diabetes insipidus. Histological examination of the pituitary gland of lesioned animals showed no abnormality of the pituitary gland. This lead Bailey and Bremer to believe that the syndrome was due to an elimination of hypothalamic control over pituitary function rather than to dysfunction of the pituitary gland itself.

The discovery of hypophysial portal vessels by Popa and Fielding in 1930 only served to heighten the controversy since Popa and Fielding believed that blood flowed from the pituitary gland upward to the hypothalamus. It wasn't until 1935 that Houssay and colleagues demonstrated downward flow in a living toad and not until 1947 that this phenomenon was confirmed in the rat by Green and Harris (see Sawyer, 1988).

G. W. Harris conducted a series of experiments in the 1940's designed to demonstrate neural control over the pituitary gland via neural factors released into portal vessels. Harris demonstrated that after lesioning of the pituitary stalk, portal vessels regenerate, and this regeneration resulted in a re-establishment of pituitary function. If the regeneration was blocked, pituitary function did not resume. With his work, Harris set the stage for the discovery of releasing and inhibiting hormones and established the concept of the brain as an neuroendocrine organ (Harris, 1961; see Sawyer, 1988).

With the discovery, isolation and characterization of a variety of releasing and inhibiting hormones that occurred from the late 1950's and throughout the 1960's, it became increasingly clear that one of the main functions of the hypothalamus is the control of the endocrine system. This control was thought to occur in two ways: 1) directly, by secretion of neuroendocrine products from the posterior lobe, or neurohypophysis, of the pituitary gland into the systemic circulation, or 2) indirectly, by secretion of neuroendocrine products into the portal plexus (of the median eminence) which drains into the blood vessels of the anterior lobe, or the adenohypophysis of the pituitary gland where these products control the synthesis and release of pituitary hormones that are, in turn, released into the systemic circulation.

This general scheme seems to apply for all vertebrate classes other than the cyclostomes (hagfish and lamprey), and teleosts (the bony fishes), which lack a median eminence. In some species of hagfish, what appears to be a primitive median eminence has been reported. In other species, it has been suggested that neural

secretions reach the adenohypophysis by diffusion. In lamprey, the situation is just as ambiguous and awaits clarification (Schreibman, 1986).

Peter has postulated that the median eminence of teleosts is actually the pituitary gland itself (Peter, *et al.*, 1990a). The pituitary gland of teleosts does have an extensive capillary bed in the neurohypophysis, fed by blood vessels that descend from the hypothalamus and contacted by hypothalamic neurons. From this capillary bed a series of capillaries pass into the adenohypophysis to vascularize adenohypophysial endocrine cells. However, these two series of capillaries do not, as far as is generally known, function in any way as a median eminence. The capillaries of the neurohypophysis do not reorganize to form a portal vessel, or vessels of any kind. In fact, neurosecretory axonal endings are believed to terminate only on the capillaries of the adenohypophysis and not on those of the primary capillary plexus in the neurohypophysis. The absence of such anatomical links between the vessels of the neurohypophysis and those of the adenohypophysis would necessarily prohibit the acknowledgement of the capillary bed in either the adenohypophysis or the neurohypophysis as a median eminence-like structure. At best, we can say that the neurohypophysis of teleosts has a much more complex structure and function than that of animals that have a median eminence.

In teleosts, the bony fishes, the lack of a median eminence is compensated for by direct innervation of adenohypophysial cells by hypothalamic neurons that terminate on or near pituitary cells (Kaul and Vollrath, 1974). The existence of such anatomical connections between the pituitary gland and discrete brain nuclei has long

implicated the central nervous system as a key regulator of pituitary function in bony fishes (Zambrano, 1972). In these animals, neuronal processes form a complex network of neurochemical signaling stations that incorporate a number of higher brain centers, receptor organs, and the pituitary gland (Schreibman, *et al.*, 1982, 1984; Schreibman and Margolis-Nunno, 1987a; Matsutami, *et al*, 1986). This network acts as a funnel, channelling a variety of environmental signals from the retina, the olfactory system and the pineal, and internal signals from the gonads and other endocrine organs, to the hypothalamus and pituitary gland to orchestrate pituitary function (Schreibman and Margolis-Nunno, 1987).

II. The genus Xiphophorus.

The genus *Xiphophorus*, is comprised of small, freshwater teleosts that have a narrow, uniform range in Mexico and Central America. *Xiphophorus helleri*, a popular and well known member of the genus, is commonly known as the swordtail because of the sword-like elongation of the tailfin rays in the males of the species. Its natural habitat is the Rio Nautla in Veracruz, Mexico but it can be found as far south as Belize and Honduras. Males range in size from 24 to 65 mm (standard length) and their sword can often double that length.

Xiphophorus maculatus is a swordless member of the genus whose range extends from the Rip Jamapa basin in Veracruz, Mexico, southward to Belize and Guatemala. Commonly known as the platyfish, these animals were classified, prior to 1960, as a separate genus, *Platypoecilus*. In natural populations, males range in

size from 18 to 45 mm (standard length) and weigh from 0.5 to 1.5 g. Females range from 14 to 45 mm in length and from 1.0 to 3.0 g in weight. Animals in laboratory populations often attain a larger size than those in the wild, with females reaching to a length of 57 mm and males a length of 49 mm.

Natural populations of swordtails and platyfish are sympatric but hybrids have never been observed in the wild. In the laboratory, however, hybrids are easily generated and have been of significant use in studies involving melanoma formation (Schartl, 1988, 1990; Schartl and Adams, 1992; Schreibman, et al., 1994).

The animals of the genus *Xiphophorus*, are valuable models for investigations into the neuroendocrine control of reproduction due to various characteristics of their reproductive physiology. Some characteristics are of significance because they are shared with higher vertebrates; these include internal insemination by copulation, internal gestation, sexually dimorphic body structure, free-swimming newborn and brain-pituitary-gonad interactions that mimic those of higher vertebrates. Other characteristics are of significance because they facilitate laboratory maintenance and study; these include sperm storage within the female for successive fertilizations and multiple broods from a single insemination, 20 to 40 animals per brood, 28 day gestation period, continuous, year-round breeding, an average life-span of 2.5 years and a predictable pattern of sexual maturation which occurs at between 14 and 26 weeks of age.

III. Brain-pituitary-gonad interactions in Xiphophorus.

X. maculatus has been the subject of extensive, longitudinal investigations that have spanned more than sixty years. It has proven to be an excellent model for studies on the complex system of interactive events and control mechanisms involving the brain, the pituitary gland and the gonads (Schreibman and Margolis-Nunno, 1987; 1989). Maturation of the reproductive system is preceded by the sequential appearance of immunoreactive (ir)-gonadotropin releasing hormone (GnRH) in three brain areas; the nucleus olfactoretinalis (NOR) first, followed, at puberty, by the nucleus preopticus periventricularis (NPP) and then the nucleus lateralis tuberis (NLT) (Fig. 1). This sequence of events, considered to be "a cascade of neural signals" results in a proliferation of pituitary gonadotropes, which in turn, results in an increase in circulating levels of gonadotropin hormones that leads to the maturation of the gonads and the onset of reproductive function.

The NOR, known in some teleosts as the terminal nerve, is a GnRH-containing neuronal system generally associated with the olfactory system. In the genus *Xiphophorus*, it consists of bilateral clusters of bipolar cell bodies that are located in the ventral telencephalon at the boundary abutting the posterior margin of the olfactory bulb. The processes of NOR neurons extend to the olfactory epithelium, the retina, the pineal, other GnRH- containing brain nuclei, and the pituitary gland (Munz *et al.*, 1981, 1982; Schreibman *et al.*, 1984; Stell *et al.*, 1984; Schreibman and Margolis-Nunno, 1987; Ekstrom *et al.*, 1988). While evidence suggests that the NOR functions as a modulator of environmental cues (retina, olfactory system, pineal; Schreibman

and Margolis-Nunno, 1987; Stell *et al.*, 1987) pheromone receptor, modulator of gonadotropin secretion, reproductive system development and physiology (Schreibman *et al.*, 1982, 1983, 1986; Schreibman and Margolis-Nunno, 1987) and reproductive behavior (Demski and Northcutt, 1983; Dulka *et al.*, 1987), the functional significance of this system is still to be definitively determined.

The NPP is a loose cluster of bipolar neurons with a rostrocaudal orientation located in the ventral telencephalon just anterior to the optic chiasm. The preoptic area of teleosts, homologous to the supraoptic and paraventricular nuclei of mammals, has long been associated with reproductive function and behavior (Davis and Kassel, 1983). Demski and colleagues showed that electrical stimulation of the pre-optic area of goldfish resulted in sperm duct contraction and sperm release (Demski, *et al.*, 1975; Demski, 1983). It was proposed that this behavioral response was mediated by a direct pathway from the pre-optic area to the spinal cord. Schreibman and co-workers demonstrated a correlation between the ontogeny of NOR and pre-optic neurons in platyfish and the onset of sexual maturation (Halpern-Sebold and Schreibman, 1983; Halpern-Sebold, *et al.*, 1986). More recently, Grober and colleagues (1994) demonstrated that changes in the number and size of pre-optic neurons in teleosts are linked to the onset of sexual maturation. The NPP and the adjacent nucleus preopticus parvocellularis are believed to contain steroid-concentrating neurons (Davis, *et al.*, 1977) as does the area ventralis telencephali pars ventralis which is located just anterior to the preoptic nucleus. In teleosts, most of the pre-optic GnRH-containing cells have been found in the NPP (Kah *et al*, 1982, Halpern-Sebold and Schreibman,

1983; Demski, 1984). It was suggested by Demski (1984) that the proximity of the GnRH neurons of the NPP and the steroid-concentrating neurons of the pre-optic area may form an important substrate for functional interactions between hormonal systems. Ir-GnRH staining of NPP neurons decreases after hypophysectomy in platyfish and this response is reversed by gonadotropin administration (Schreibman, *et al.* 1983). This data is consistent with the concept of negative feedback exerted by gonadal steroids, on GnRH secretion, via NPP neurons.

The NLT, known to be the source of hypophysiotropic neurons, is located in the ventral hypothalamus and has been arbitrarily divided into three rostrocaudal divisions, the pars anterioris, the pars posterioris, and the pars inferioris (Peter and Gill, 1975). It is populated predominantly by bipolar neurons with a dorsoventral orientation. Ir-GnRH containing neurons have been found in the pars posterioris of the NLT in several teleostean species (Halpern-Sebold and Schreibman, 1983; Kah, *et al.*, 1982; Demski, 1984; Batten, 1990). This nucleus is known to mediate steroid feedback control of gonadotropin release via control of GnRH secretion (Demski, 1984). This nucleus responds in the same manner as the NPP to hypophysectomy and gonadotropin replacement (Schreibman, *et al.*, 1983). It has been suggested that the GnRH neurons of the NLT are also steroid-concentrating neurons (Demski, 1984). This nucleus, homologous to the arcuate and ventromedial nuclei of mammals, is believed to receive extensive neural input from the ventral telencephalon. In platyfish, the appearance of ir-GnRH in NLT neurons heralds the proliferation of pituitary gonadotropes and the subsequent maturation of the gonads.

As in other vertebrates, the pituitary gland of teleosts is comprised of a neural component, the neurohypophysis and an epithelial component, the adenohypophysis. The neurohypophysis contains the axonal endings of neurons whose cell bodies are located in the hypothalamus, as well as in extra-hypothalamic regions of the brain, and whose axons course downward to the pituitary gland through the infundibular stalk. Neurohypophysial tissue can be seen interdigitating with adenohypophysial tissue in the body of the gland.

The adenohypophysis of the teleost pituitary is distinguished by the regional distribution of pituitary cell types according to their physiological function (Fig. 2). It is divided into three rostrocaudal divisions, the rostral pars distalis (RPD), the caudal pars distalis (CPD) and the pars intermedia (PI) (Schreibman, *et al.*, 1973). In neonatal platyfish, the gland is populated by all the cell types found in the adult gland with the exception of the zone of gonadotropes in the ventral CPD. Gonadotropes, in the neonatal and immature gland are found only in the extreme lateral sections of the CPD and in small clusters in the PI. As sexual development proceeds, the gonadotropes of the CPD proliferate under the influence of GnRH from the brain, to form the thick gonadotropic zone that girdles the outer boundary of the ventral CPD of the adult (Schreibman and Margolis-Nunno, 1987).

In the genus, *Xiphophorus*, the age at which the above events occur and sexual maturity ensues, is determined by an allelic series at a genetic locus, on the sex chromosome, termed *P*. In genetically defined stocks of *X. maculatus*, nine *P* alleles have now been identified and they are known to control not only the age, but also the

size at which sexual maturity will occur (Kallman, 1989). The conditions under which these animals are raised will have a bearing on the process of sexual maturation. However, given similar environmental conditions, these animals will mature at a time dictated by their *P* allele composition.

The various *P* alleles are known to be closely linked to different pigment genes which control body coloration. Therefore, body markings of platyfish serve as phenotypic indicators of the alleles present at the *P* locus. Using these phenotypic markers, it is relatively easy to set up matings to generate offspring that will mature at predictable ages. The genetic cross presented below, indicating body pigment markings and their corresponding *P* alleles, illustrates one of the routine matings used to generate offspring:

P_1 $X^{N}(P^{5})$ $X^{Sp}(P^{1})$ **X** $X^{Sp}(P^{1})$ $Y^{Sr}(P^{2})$

F_1	$X^{Sp}(P^{1})$ $X^{Sp}(P^{1})$	early maturing females
	$X^{N}(P^{5})$ $X^{Sp}(P^{1})$	late maturing females
	$X^{Sp}(P^{1})$ $Y^{Sr}(P^{2})$	early maturing males
	$X^{N}(P^{5})$ $Y^{Sr}(P^{2})$	late maturing males

N= 2 - 3 very large, black markings on the animals' flanks
Sp= animal's flanks speckled with very small blacks markings
Sr= animal's flanks appear to have very thin black stripes extending from the operculum to the tail fin.

The functional significance of this genetic cross is that it enables the investigator to effectively uncouple sexual maturation and chronological aging. The offspring generated are siblings, bred and reared under identical conditions, yet they will reach maturation at significantly different ages. For example, males with the P^1P^2 (SpSr), will mature at 14+/-0.3 weeks of age and 21.4+/-0.3 mm standard length while their sibling males, with the P^5P^2 genotype (NSr), will mature at 33.2+/-0.8 weeks and 35.2+/-0.5 mm standard length. In addition, these genetically defined stocks of platyfish are highly inbred, reducing variability. A different pattern of inheritance is present in swordtails but the precise details of this pattern are still unknown.

The anal fin of male platyfish undergoes an androgen-dependent transformation that converts the fin into an intromissive organ, known as the gonopodium, that serves to introduce sperm to the female during copulation (Grobstein, 1948). This transformation occurs in six well-defined stages that have been linked to specific events occurring in the brain, pituitary gland and gonad as these animals approach sexual maturity. The stage of anal fin development, therefore is an excellent external, *in vivo* marker betraying internal developmental events (Kallman and Schreibman, 1973; Schreibman and Kallman, 1977; Schreibman, *et al.*, 1982a). The progress of sexual maturation in females, who do not, of course, exhibit gonopodial development, can only be evaluated by post-mortem examination of the gonads. Fish with the early maturing *P* allele composition, will exhibit the following significant stages of gonopodial development:

a. Neonatal (two days to 0.5 mos old)

The anal fin and the gonad, of fish at this stage, are undifferentiated. Males are indistinguishable from females. There are no ir-GnRH containing perikarya or fibers in the brain. The pituitary gland contains all the cell types of the adult with the exception of the gonadotropes of the ventral caudal pars distalis. The gonad contains undifferentiated cells.

b. Gonopodial Stage One (occurs at approximately 5 weeks of age)

The first stage of anal fin metamorphosis is marked, in males, by a slight elongation of the most ventral anal fin rays. This occurs between five and seven weeks of age in both early and late maturing Rio Jamapa 163 stocks and is linked to increasing androgen levels. The gonads have begun their development. The ovaries of females at a comparable stage of development would contain white, immature oocytes while the testes become somewhat larger and slightly opaque. In stocks that possess *P* alleles for late maturation, the animals remain in this stage, while their early maturing siblings proceed with the development process.

In the brain at this stage, ir-GnRH is found in perikarya and axons of the NOR. A few fibers of the NPP also demonstrate an immunoresponse to anti-GnRH, but no perikarya of the NPP or the NLT are found to contain ir-GnRH.

The pituitary gland of both males and females has a thin gonadotropic zone in the peripheral caudal pars distalis consisting of a small number of scattered

chromophobes. Ir-betaGTH is found in the lateral caudal pars distalis (CPD) and in the pars intermedia (PI).

c. Gonopodial Stage Two (occurs at approximately 1.5 to 2 mos of age)

This stage of anal fin metamorphosis is marked by a pronounced elongation of the ventral anal fin rays. This stage marks the onset the maturational process known as puberty. Stages three to five quickly follow, during which time gonadal development and sexual maturity proceed rapidly, following the formation of a fully developed gonadotropic zone in the pituitary gland. Increased numbers, and activation, of pituitary gonadotropes result in rising circulating levels of GTH. The testis become increasingly opaque as sperm production proceeds. In the ovaries, oocytes enlarge and some yolk deposition may occur.

At stage two, ir-GnRH is found in perikarya and fibers of both the NPP and NLT, as well as in those of the NOR. This event is associated with an increase in the size of the perikarya of these brain centers, and with an increase in the size and prominence of the nuclei and nucleoli of the immunoresponsive neurons.

Ir-GnRH and -betaGTH begin to appear in the gonadotropic cells of the ventral caudal pars distalis of the pituitary gland. The rapid proliferation of these gonadotropes in the pituitary gland leads to the formation of a thick band of gonadotropic cells that girdles the caudal pars distalis.

d. Gonopodial Stage Six (occurs at approximately 3 to 3.5 mos of age)

This is the final stage of anal fin metamorphosis and it is marked by the transformation of the anal fin into a fully developed gonopodium capable of internal fertilization. This stage is an indication that the completion of gonadal development and sexual maturity are at hand. The testes of fish at this stage are large, well-developed and demonstrate all stages of spermatogenesis. The ovaries contain many large, ripe, yolky oocytes.

In stage six animals, there is a perceptible increase in the number of ir-GnRH containing perikarya and fibers in the NPP, compared to earlier developmental stages. The NOR and the NLT do not demonstrate a similar change.

The gonadotropic zone of the pituitary is thick and fully developed. Ir- GnRH and -betaGTH are found in the gonadotropes of the ventral and lateral caudal pars distalis and in cells of the pars intermedia.

IV. Regulation of Reproductive Function

A. Neuroregulatory Peptides

A key mystery that remains unsolved in our understanding of the reproductive physiology of the members of the genus *Xiphophorus*, is the nature of the neural signals that impact on the GnRH-containing neurons of the brain. These signals could initiate the cascade that results in the onset of puberty, perfect the timing of the cascade and orchestrate the neurophysiological and physiological events involved in successful reproductive function.

A wide variety of distinct neurochemicals have gained notoriety as purported neuroregulatory and hypophysiotropic factors involved in reproductive physiology. Among the more significant are:

1. FMRF-amide (Phe-Met-Arg-Phe-NH_2)

A tetrapeptide that was first discovered as a cardioexcitatory peptide in mollusks (Price and Greenberg, 1977). A FMRF-amide-like peptide has been shown to coexist with immunoreactive (ir)-GnRH in the neurons of the NOR (Stell *et al.*, 1985). Furthermore, Rama Krishna and colleagues (1992) demonstrated a direct innervation of the pituitary gland by fibers from neurons of the nervus terminalis of catfish that were shown to contain a FMRF-amide-like peptide.

2. Galanin (GAL)

A widely studied, 29 amino acid peptide, originally isolated from porcine small intestine (Tatemoto *et al.*, 1983), has been shown to be a major modulator of central nervous system function, influencing plasma hormone levels (Rokaeus, 1987; Crawley and Wenk, 1989). Holmqvist and Ekstrom (1991) and Batten *et al.*, (1990a) described the distribution of GAL-like immunoreactivity in the brain and pituitary gland of several teleost species. These studies, bolstered by the evidence in mammals (Davis *et al.*, 1987; Melander *et al.*, 1986), indicate that GAL, or a GAL-like peptide, may be involved in pituitary function.

3. Neurotensin (NT)

A thirteen amino acid peptide that was first isolated from bovine hypothalami (Carraway and Leeman, 1973, 1975). An NT-like peptide has been found in neurons and endocrine cells of the central and peripheral nervous systems and in the gastrointestinal tract of mammals (see Polak and Bloom, 1982; Reinecke, 1985) as well as in other vertebrates and invertebrates (Carraway, *et al.* 1982). There is also evidence to suggest that NT is implicated in regulating pituitary function (Rivier *et al.*, 1977; Maeda *et al.*, 1978; Vijayan and McCann, 1979; Enjalbert *et al.*, 1982; McCann *et al.*, 1982). Batten *et al.*, (1987) localized an NT-like peptide in the hypothalamus and pituitary gland of several species of bony fishes.

4. Neuropeptide Y (NPY)

A tyrosine-rich, thirty-six amino acid peptide that was first isolated from porcine brain (Tatemoto *et al.* 1982). In mammals, NPY neurons are reported to be widely distributed throughout the brain, with the highest concentrations occurring in the hypothalamus and in the median eminence (Gray and Morley 1986). Recent evidence suggests that this peptide may play a role in reproductive physiology by influencing the release of luteinizing hormone-releasing hormone (LHRH) from nerve cell terminals (Woller and Terasawa 1991; Kaynard *et al.* 1990) or perhaps even through a direct effect on pituitary gonadotropes (Crowley and Kalra 1988). Other reports have linked this peptide to feeding and sexual behavior (Morris and Crews, 1990), and to growth hormone (McDonald, *et al.*, 1985; Peng, *et al.*, 1990).

5. Dynorphin (DYN)

Dynorphin is an opioid peptide cleaved from a precursor, prodynorphin. Structurally, DYN is a C-terminally extended form of Leu^5-enkephalin, but it is the product of a different gene (Delitala 1991). It has been isolated from the brain, pituitary, adrenals, spinal cord, and reproductive organs of mammals (Stampinato *et al.* 1991) with the highest concentrations occurring in the posterior pituitary and the hypothalamus. DYN is known to bind to the kappa-type opioid receptor (Olson and Welch 1991).

Studies involving NPY and DYN in non-mammalian vertebrates have been interesting, particularly among teleostean species (Noe *et al.* 1989; Pontet *et al.* 1989; Batten *et al.* 1990; Peng *et al.* 1990; Danger *et al.* 1991). These peptides have been implicated in a variety of physiological events in mammals. This has created a great deal of interest as to their physiological significance in other, non-mammalian vertebrates.

6. Substance P (SP)

Substance P is an undecapeptide postulated to be a neurotransmitter or neuromodulator in both the peripheral and central nervous systems (Pernow, 1983; Aronin, *et al.*, 1986). Investigations have indicated that SP has a functional role in anterior pituitary regulation and when administered *in vivo* can alter luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin (PRL), and growth hormone (GH) secretion (Kato, *et al.*, 1976; Rivier, *et al.*, 1977; Chihara, *et al.*, 1977;

Vijayan, *et al.*, 1979). In mammals SP-like immunoreactivity has been found in the anterior pituitary and in regions of the hypothalamus associated with pituitary regulation, and it has, itself, been shown to be susceptible to gonadal steroid regulation (Brown, *et al.*, 1990). There is a paucity of information on the presence and activity of this peptide in non-mammalian vertebrates.

7. Bradykinin (BK)

Bradykinin is a nine amino acid peptide that is part of the kallikrein-kinin system in mammalian cardiovascular regulation. In mammalian systems, it has been shown to effect hormone release from cultured pituitary cells (Kuan, *et al.*, 1990). It has recently been isolated in turtles (Conlon, *et al.*, 1990) but there is still little information available on this peptide in lower vertebrates.

8. Vasoactive Intestinal Peptide (VIP)

Vasoactive intestinal peptide is a 28 amino acid peptide originally isolated from porcine duodenum (Said and Mutt, 1970). VIP has been demonstrated in peripheral tissues such as the gastrointestinal, genitourinary and respiratory systems (Costa, *et al.*, 1980; Larrson, *et al.*, 1977; Larrson, *et al.*, 1978) and has been immunocytochemically detected in the central nervous system (Fuxe, *et al.*, 1977). It has been found in the hypothalamus of birds (Ball, *et al.*, 1986) as well as rats (Besson, *et al.*, 1979) and it has been suggested that the immature rat gonad is under direct central nervous system control mediated by VIP-containing fibers (Ahmed, *e t al.*, 1986). In fish, there is

some evidence to indicate that VIP may be involved in pituitary function (see Grau and Helms, 1990).

This report seeks to extend the existing information on regulatory neuropeptides through a longitudinal study of the distribution of immunoreactive-FMRF-amide, -galanin, -neurotensin, -neuropeptide Y, -dynorphin, -substance P, -bradykinin and -vasoactive intestinal peptide in the brain and pituitary gland of *Xiphophorus* from birth to sexual maturity. We combine the investigative advantages of our *Xiphophorus* model with those of immunohistochemical techniques to study the distribution of these neuropeptides in the brain and pituitary gland at various significant stages of sexual development in an attempt to elucidate the relationship between these regulatory neuropeptides and the process of sexual maturation.

B. Gonadotropin Releasing Hormones

Phylogenetic studies have demonstrated that gonadotropin releasing hormone (GnRH) is conserved as a family of eight decapeptides that share identical terminal groups (Table 1) (Sower, *et al*, 1993). The existence of multiple forms of GnRH in the brain and pituitary gland of a single species has been demonstrated in birds (King and Millar, 1982, 1982a,; Powell, *et al.*,1987), reptiles (Lovejoy, *et al.*, 1991), amphibians (King and Millar, 1986; Jones, 1987; Sherwood, *et al.*, 1993), and fish (King and Millar, 1985; Lovejoy, *et al.*, 1992; Ngamvongchon, *et al.*, 1992). In mammals, only one form of GnRH has been characterized, yet the existence of

another variant form has been suggested (Stopa, *et al.*, 1988). Among the teleostean fishes, studies have suggested that the major form of GnRH is chromatographically and immunologically identical to salmon GnRH (sGnRH) (Sherwood *et al.*, 1984; King and Millar, 1985; Powell *et al.*, 1986). Other forms of GnRH reported to be present in teleosts include chicken II GnRH (chIIGnRH) (Powell *et al.*, 1986), chicken I GnRH (chIGnRH) (King and Millar, 1985), mammalian (mGnRH) (Schreibman, *et al.*, 1979), dogfish GnRH (Lovejoy, *et al.*, 1992) and catfish GnRH (Ngamvongchon, *et al.*, 1992). Barnett and colleagues (1982) reported the presence of three different LHRH-like substances in the teleost brain.

The genetic derivation of multiple forms of the GnRH molecule has not been elucidated. DNA coding sequences have been determined for the mGnRH prohormone in several mammalian species (Seeburg and Adelman, 1984; Adelman, *et al.*, 1986; Mason, *et al.*, 1986) and for the chicken I precursor in chicken (Dunn, *et al.*, 1992). More recently, the cDNA clones encoding the precursor for sGnRH were isolated from two species of bony fish (Bond *et al.*, 1991; Klungland, *et al.*, 1992). Grober and co-workers (1992) have isolated and cloned the cDNA of one form of GnRH in the midshipman, *Porichthys notatus*, and have evidence for the existence of a gene encoding another form of the molecule in the same species. Attempts to isolate the precursor chIIGnRH gene in either chicken or in the numerous species in which this peptide has been found, have not so far been successful, however, these efforts have established that different forms of GnRH are encoded by different genes and are not processed from a single precursor (King and Millar, 1993). King and Millar (1993)

have hypothesized that multiple forms of GnRH arose from gene duplication that resulted in different genes. There is also evidence that multiple forms coexist within a single cell (see Sherwood, 1993). These data would indicate there is a functional significance to the presence of multiple forms of GnRH in a single species and that variant forms are mediating different aspects of neuroendocrine function (Magliulo-Cepriano, *et al.*, 1994).

C. Gonadotropin Hormones

GnRH is the key regulator of the synthesis and release of pituitary gonadotropin (GTH). In mammals two forms of GTH have long been known to be present, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Until fairly recently, it was thought that in teleosts only one form existed and was responsible for the full range of gonadal functions and actions. This concept was refuted with the isolation and characterization of two distinct forms of beta GTH in salmon, GTH I and GTH II (Suzuki *et al.*, 1988; Swanson *et al.*, 1991), that displayed different physiological activities and were subject to differential regulation (Suzuki, *et al.*, 1988a; Naito *et al.*, 1991).

Now that the presence of multiple forms of both GnRH and GTH has been established in teleosts, the nature of the relationship and interactions between these peptides becomes an important question to address. This report seeks to utilize the natural advantages of the platyfish model to help elucidate the physiological and

anatomical relationship that exists between the multiple forms of GnRH and GTH during the development of the reproductive system of teleosts, and to evaluate the possibility of physiological interactions between GnRH and regulatory neuropeptides and neurotransmitters.

D. Amino Acid Neurotransmitters

1. Gamma-aminobutyric Acid

Gamma-aminobutyric acid (GABA) is an important hypothalamic amino acid neurotransmitter in mammals and other species. It is a ubiquitous and abundant component of synaptic input to a variety of hypothalamic nuclei (Decavel and van den Pol, 1990). Made from a glucose precursor, GABA has been designated as an inhibitory neurotransmitter mediating a wide variety of neurophysiological functions throughout the brain. While the vast majority of GABA investigations have utilized mammalian systems, some studies have been conducted in goldfish (Kah, *et al.*, 1987; 1990; 1992; Martinoli, *et al.*, 1990). These studies indicate that GABA may have a role in regulating pituitary function, perhaps in the stimulation of GTH release from pituitary gonadotropes. This would be in contrast to the inhibitory actions typically associated with this neurotransmitter, however, there have not been many studies in other teleosts nor have there been follow-up studies in goldfish to determine how GABA brings about this stimulation of GTH release. The full story of GABA actions and interactions in teleosts remains to be elucidated.

2. Glutamate

Glutamate (GLU) is an excitatory amino acid neurotransmitter that, again, has received a lot of attention from investigators utilizing mammalian systems but relatively none from those studying non-mammalian species. A small number of studies have been conducted in goldfish with promising results (Kah, *et al.*, 1983; Sloley, *et al.*, 1992). These studies indicate there is GLU innervation of the teleost pituitary and that this amino acid affects GTH secretion.

This study will attempt to localize GABA and GLU immunoreactivity in the brain and pituitary gland of *Xiphophorus*, and to determine the likelihood of their involvement in pituitary functions related to reproduction.

E. Serotonin

Serotonin was first crystallized from mammalian blood in 1949 (see Page, 1976). Its name was derived from the observation that after blood clots, the resultant serum had the ability to increase vascular tone. We now know that, serotonin, released from aggregating blood platelets, causes the noted vascular "toning" effect of the serum. Produced from the precursor amino acid tryptophan, serotonin is widely distributed in nature and is found in the neural tissues of many species of vertebrates and invertebrates, in fact, serotonergic neurons are the first to appear during fetal development in rats (Jacobs, *et al.*, 1990).

In fish, studies have linked serotonin to various pituitary functions such as somatotrope physiology (Somoza and Peter, 1991), secretion of melanophore-stimulating hormone (Olivereau, 1978a) prolactin secretion (Olivereau, 1978b) and GTH release (Somoza, *et al.*, 1988; Somoza and Peter, 1991). Recent studies by Khan and Thomas (1992; 1993) provide strong evidence that in fish, serotonin is involved in the secretion of pituitary GTH.

In sexually mature platyfish, ir-serotonin has been localized in the brain and pituitary gland (Margolis-Kazan, *et al.*, 1985) and changes in the cytochemical distribution of the immunoreactivity with age suggest that this neurotransmitter may be involved in reproductive system senescence (Margolis-Nunno, *et al.*, 1986).

This report seeks to extend the available information on the distribution of ir-serotonin in platyfish to earlier stages of development than those encompassed by the previous studies. Perhaps the same neurotransmitter that is suspected of being involved in the changes associated with senescence, is also implicated in the events leading to sexual maturity.

F. Dopamine

Dopamine, a catecholamine produced from tyrosine in the same biochemical pathway that yields norepinephrine and epinephrine, is found in both peripheral tissues and in the central nervous system where it serves as a neurotransmitter in several important neural pathways. It has been found in numerous species and has been assigned numerous functions. It has been associated with GnRH and GH release

(Peter, *et al.*, 1986; Yaron and Levavi-Sivan, 1990; Wong, et al., 1993) and with prolactin inhibition (Grau and Helms, 1990) in fish.

This study will seek to confirm the involvement of dopamine in pituitary and reproductive physiology through an anatomical mapping of dopamine immunoreactivity in the platyfish brain and pituitary gland.

G. Steroid Feedback

Steroid concentrating neurons have been found in the NPP and the NLT of the teleost brain. It is believed that these neurons react to circulating levels of steroids by modulating GnRH secretion and thus, play an important role in the maturation and maintenance of reproductive system physiology. In combined immunocytochemical and autoradiographic studies on platyfish, Kim and colleagues (1979) found cells which concentrated estrogen and were also immunoreactive to antiserum to ovine luteinizing hormone. They found these cells in the caudal pars distalis and the pars intermedia of the platyfish pituitary gland. Using a fluorescent steroid hormone-conjugate, Schreibman and co-workers (1982b) corroborated the results of the earlier study and went further to demonstrate that the hormone-conjugate was localized in the cytoplasm and nucleus of the pituitary cells. However, neither study demonstrated steroid localization or binding sites in the brain.

The neural events orchestrating the maturation of the reproductive system in *Xiphophorus* are very probably influenced by activity in the developing gonad. To fully understand the role various brain structures play in the process of sexual

maturation, it is essential to elucidate those structures which are most sensitive to this gonadal influence. This sensitivity is likely to be manifested by the presence of steroid receptors associated with neurons that serve in feedback control mechanisms. A knowledge of where steroid receptors are located in the *Xiphophorus* brain would make a significant contribution to this area of interest.

Thus, the neuroendocrine control of reproduction in teleosts is multifactorial and highly complex. The unraveling of the myriad interactions that result in the development and functioning of the reproductive system has been the object of decades of scientific study and will, no doubt, continue to be so for very many more. This report seeks to extend the existing information by addressing the following specific questions:

1. Do the regulatory factors, listed above, occur in *Xiphophorus*?
2. What is the immunocytochemical distribution of those neuropeptides and neurotransmitters that prove to be present in the *Xiphophorus* brain and pituitary gland and does this distribution change as the animal proceeds from birth to sexual maturity?
3. Is there a developmental timetable according to which these neural peptides make their appearance in the *Xiphophorus* brain and pituitary gland?
4. Will exogenous administration of these peptides affect the process of growth and development?

6. What area(s) of the brain and pituitary gland might serve as centers for feedback control by gonadal steroids?

7. How does the information derived from the answers to the above questions contribute to our understanding of the genetic and neuroendocrine control of reproductive system development and function?

Materials and Methods

I. Animals

One hundred and sixty (100 males, 60 females) platyfish, *Xiphophorus maculatus,* derived from genetically defined stocks [JP163A or JP163B] at the New York Aquarium Genetics Laboratory were utilized in this study. For the investigations involving FMRF-amide, neurotensin, and galanin, in addition to platyfish, we studied sixty marigold wagtails (40 males, 20 females), and thirty swordtails (20 males, 10 females), *X. helleri*, from the Helleri-3 line at the Ruhr University in Bochum, Germany. The marigold wagtails are a variety of *X. maculatus* of commercial origin but bred, by brother to sister matings, and reared in our laboratory for several generations. The JP163 platyfish and the swordtails reach sexual maturity at 12-16 weeks of age, while the marigold wagtail platyfish are sexually mature at 7-8 weeks of age. Except where indicated, results reflect data gathered in JP163 platyfish.

All animals were kept in tanks containing aged tap water, gravel, snails and live plants, at an average temperature of 21-22 degrees centigrade and a 16L:8D photoperiod. They were fed thrice daily a diet of fresh brine shrimp nauplii and a liver-cereal paste, supplemented by commercially prepared flake food.

II. Tissue processing

Animals were sacrificed at the following ages: 1 - 2 days (neonates), 0.5 mos, 1.5 mos, 3.0 mos, and 10 mos. Fish aged 1.5 mos or older were sacrificed by decapitation after anesthetization with 0.04% tricaine methane sulfonate (MS 222) (Sigma, U.S.A.). Heads and bodies were placed in Bouin solution and fixed under vacuum for 24 to 48 hrs. Younger fish were sacrificed by whole organism immersion in Bouin solution after light anesthesia, and then placed under vacuum. Animals to be processed for immunocytochemical staining with antisera conjugated to glutaraldehyde were fixed in 3% glutaraldehyde overnight at 4 degrees C. Specimens were then decalcified (S/P Decalcifying Solution, Baxter, McGaw Park, Ill.), dehydrated in ethanol and butanol (Zirkle series), and embedded in polyfin (Triangle Biomedical Supplies, Durham, N.C.). Five micrometer thick, serial, sagittal or transverse sections were mounted on gelatin-coated (subbed) microscope slides.

III. Immuno-staining protocols

Immunohistochemical procedures utilized the unlabeled antibody peroxidase-antiperoxidase (PAP) method (Sternberger *et al.*, 1970) as modified for our material (Margolis-Kazan and Schreibman, 1981). Polyclonal rabbit antibodies were utilized to map the immunocytochemical distribution to FMRF-amide, GAL, NT, NPY, DYN, SP, BK, VIP, mammalian GnRH, salmon GnRH, chicken II GnRH, lamprey GnRH, serotonin, dopamine, glutamate, GABA, and androgen receptors in the brains and pituitary glands in animals in each of the age groups listed above. Antibodies were diluted in TRIS (Sigma, St. Louis, Mo.) buffered saline (pH 7.6) and used as primary antisera (dilutions and specifications of antisera used are given in Table 2). The antisera were well-characterized by the supplier specifically for suitability in immunocytochemistry procedures. Additionally, we ascertained the specificity of the antisera by standard Ouchterloney reactions and by absorption of the antisera with their homologous antigen (0.01 mg antigen per 0.001 mg antisera, in final dilution), which, in all cases, eliminated the immune reaction. The initial step in the study of the variant forms of GnRH was to conduct an absorption study in which each antiserum was absorbed with homologous and heterologous antigens. In all cases absorption with the homologous antigen resulted in the elimination of the immunoreaction. In cases where absorption of the antisera with the heterologous antigen decreased the staining characteristics of the antisera, the absorbed antisera were used for the final study. For results of the absorption studies, see Table 3.

IV. Colocalization of two antigens

Each of the immunoreactive (ir)-factors under investigation was tested for colocalization with pituitary hormones utilizing antisera listed in Table 2. In addition, the following were tested for colocalization: ir-FMRF-amide and ir-GnRH, ir-FMRF-amide and ir-NPY, ir-NPY and ir-GnRH. ir-GAL and ir-arginine vasotocin (AVT), ir-GAL and ir-GnRH.

To colocalize two antigens in one tissue section or to compare the distribution of antigens, we used two approaches: 1) incubation of adjacent serial sections with antibodies against different antigens, and 2) a double staining method for colocalization of two antigens in one tissue section using the avidin-biotin method of immunocytochemistry (Vectastain-ABC and ABC-AP, Vector Laboratories, Burlingame CA.). The first primary antibody was linked to a peroxidase enzyme marker and visualized by incubation (10 min, room temperature) with 0.025% 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, Mo.) in TRIS buffer containing 0.0125% hydrogen peroxide. The first primary antibody was removed from the section by incubation (30 min, room temperature) in glycine buffer (pH 2.2). The second primary antibody was linked to an alkaline phosphatase enzyme marker and visualized by use of Vector's alkaline phosphatase Substrate Kit III (blue). Control procedures included:

a. the replacement of the primary antibody with normal rabbit serum,

b. for double-staining controls, both the first and second primary antibodies were alternately replaced to ascertain that reagents linked to the first antigen were not

reacting with reagents used to localize the second antigen or that the product of the first reaction was not modified by the reagents of the second reaction,

c. the elimination of sequential steps in the immunocytochemistry procedure

d. the absorption of the primary antisera with its homologous antigen, and, where indicated, with structurally similar heterologous antigens.

In each immunocytochemistry run, 20 to 30 slides were processed which included representatives from each age group. All comparisons were made on slides processed simultaneously. No comparisons were made between ABC and PAP processed slides. Comparisons based on staining intensities were made only between regions in the same tissue sections and on the same slide.

Representative slides were stained with Masson's trichrome and Periodic acid-Schiff to aid in graphical and cytological analysis. Atlases of the brains of platyfish (unpublished) and molly, *Poecilia latipinna*, (Batten *et al.*, 1990) were used to determine sites of localization. Camera lucida drawings were utilized to compare regions of antigenic and tinctorial responses.

V. Exogenous administration of Neuropeptide Y

Twenty-two JP163 platyfish were selected for this study. Ten immature siblings, 4 weeks old were divided into two groups, five experimental and five controls. The remaining twelve, adults and also siblings, consisted of six females and six males, 17 weeks old, which were equally divided into experimental and control groups. All experimental animals were given five intraperitoneal injections per week

(1 microgram/0.5 gram body weight) of neuropeptide Y (Cambridge Research Biochemicals, Wilmington, DE.) in 0.65% saline. Control animals were given a 0.65% saline solution. Injections took place at midmorning. All animals were lightly anesthetized, standard length was determined, gonopodial development evaluated. Animals were then injected and returned to their tanks. Percent difference in measured length was determined on a week to week basis. Adults were given a total of 10 injections then were sacrificed, at the age of 19 weeks, by decapitation after anesthesia and processed for immunocytochemistry. The immature animals were given a total of 25 injections before sacrifice, at age 10 weeks, by decapitation after anesthesia. Specimens were then processed for histological examination and immunocytochemistry according to the protocol outlined above. Sections of brain and pituitary were immunostained with antisera to NPY, GnRH, GH, GTH, and FMRF-amide using Vectastain-ABC and DAB. Stained sections were evaluated and immunoresponsive pituitary cells counted by viewing the sections on a grid and counting every immunoresponsive cell that displayed a nucleus from the anterior to the posterior end of the gland in every third section. Statistical significance was determined by use of the student T test with $p < .05$ (95% confidence range) taken as significant.

Results

I. Regulatory Neuropeptides

The reader is referred to Table 4 and to Figs. 3 and 4 for a summary of the distribution of the ir-regulatory neuropeptides discussed below.

FMRF-amide

Immunoreactive-FMRF-amide was localized in perikarya and processes of the neurons of the nucleus olfactoretinalis (NOR) in adults of all three stocks (Fig. 6). The processes formed tracts that extended through the preoptic area (Fig. 7, 8) to the medial nucleus lateralis tuberis (NLT), where they arborized along a dorsal-ventral axis (Fig. 4a). In the NOR, there is a subpopulation of neurons in which ir-FMRF-amide and ir-gonadotropin releasing hormone (GnRH) coexist. There are also neurons within this nucleus that appear to contain only one or the other of these two peptides. It appears that colocalization of these peptides also occurres in neural processes in the preoptic area and in the NLT. The system of ir-FMRF-amide containing fibers was far more extensive than that of GnRH, particularly in the dorsal telencephalon. Fibers containing ir-FMRF-amide were also observed in the optic tectum and in the nucleus recessus posterioris.

In the brain of all three *Xiphophorus* stocks studied, the immune response increased in both the intensity of the staining and in the number of cells stained, as the

animals matured. Two day old fish demonstrated ir-FMRF-amide in NOR perikarya but the immune reaction in neural processes of NOR neurons, was negative. Fish 0.5 mos old showed sparse, pale ir-tracts extending from the NOR into the NPO. There were a few scattered, ir-tracts in the NLT, as well, but none in the NPP. The By 1.5 mos of age, at which time all fish are in the initial stages of sexual maturation, there were numerous ir-fibers traversing the region of the brain between the NOR and the nucleus preopticus periventricularis (NPP), in the nucleus preopticus (NPO) and throughout the NLT.

In the pituitary gland, ir-FMRF-amide was seen as granules in the neurohypophysis, concentrated along the basement membrane separating the neurohypophysis and the adenohypophysis, and in the cells of the rostral pars distalis (RPD) (Fig. 3). The immune response in the pituitary was of low intensity, in all age groups, suggesting a low level presence of the peptide.

Galanin

Animals aged 2 days and 0.5 mos did not demonstrate an immune reaction to anti-GAL in the brain.

In all animals aged 1.5 mos and older, ir-GAL containing axonal tracts and terminals were localized in the NPO, the NPP, in the ventral telencephalon between the NOR and the NPP and in the posterior NLT (Fig. 4b). Neural tracts containing ir-GAL traversed the NPO in an anterior-posterior orientation. In the NPP, ir-perikarya formed a loose cluster of approximately thirty neurons that extended from

the ventral region of the telencephalon upwards towards the NPO. Immunoreactive perikarya were also localized in the posterior NLT in one or two smaller, tighter clusters of approximately four neurons.

In the pituitary gland, two day old animals of all three stocks demonstrated an immune response to anti-GAL in the CPD and the neurohypophysis of the pituitary gland. Marigold wagtails of this age group also showed a response in the RPD and in the PI of the gland. Ir-GAL was localized in cells in all three regions of the adenohypophysis (Figs. 3, 9) of animals aged 0.5 mos and older. Ir-GAL appears to colocalize with ir-growth hormone (GH) in the caudal pars distalis (CPD) and with ir-somatolactin (SL) (Fig. 10) in the pars intermedia (PI). Ir-GAL was found in the neurohypophysis directly dorsal to, but not in contact with, the pituitary gonadotropes of the CPD. Ir-GAL colocalized with ir-prolactin (PRL) in the RPD.

Neurotensin

In animals aged 0.5 mos, ir-NT was localized only in tracts and terminal fields in the anterior NLT (Fig. 4c). In the brain of all animals aged 1.5 mos and older, ir-NT was demonstrated within perikarya of the ventral anterior NLT and within tracts and terminal fields throughout the anterior NLT (Figs. 4c, 11).

Ir-NT was seen within cells of the CPD, RPD and PI (Fig. 3) of all animals aged 1.5 mos and older. Immunoreactivity in the CPD was limited to a band of cells that border the neurohypophysis (Fig. 12). Antisera to pituitary hormones did not demonstrate any colocalization within these ir-NT containing cells. Also in these

animals, at the border of the neurohypophysis and the dorsal RPD an intensely stained, thin band of immunoreactivity was observed Fig. 12). It was not clear if this immunoreactivity was localized within cells of the RPD or along the basement membrane that separates the adenohypophysis from the neurohypophysis. This immunoreactivity abutted a region of the adenohypophysis that is populated by ir-adrenocorticotropin (ACTH) containing cells.

In the pituitary gland of all sexually mature fish (3 mos), ir-cells in the PI formed a dense band at the posterior tip of the gland (Fig. 13). In younger animals, this ir-band of cells was absent and only scattered ir-cells were seen in this region of the PI.

There were distinct differences among the three stocks of *Xiphophorus* studied in the distribution of ir-NT in the pituitary gland of animals aged less than 1.5 mos. Marigold wagtails demonstrated an immune response to anti-NT in all age groups studied. In the JP163 stock of platyfish and in the swordtails, immunoreactivity was not observed in the two day old age group. The swordtails did demonstrated low levels of immunoreactivity in the pituitary glands of 0.5 mos old animals while the JP163 platyfish did not.

Neuropeptide Y

Ir-NPY was localized in bilateral clusters of four to twelve bipolar, ovoid-shaped perikarya in the NOR (Fig. 14). Ir-NPY was also seen in the processes extending from these perikarya, forming tracts that extended dorso-posteriorly towards

the NPO and the NPP (Fig. 4d). In neonatal animals, immunoreactivity was limited to perikarya of the NOR and did not extend into the processes as it did in all other age groups studied.

In the telencephalon, ir-NPY was localized in bilateral clusters of approximately twenty spherically-shaped perikarya. These perikarya appear to be located in the area ventralis, pars lateralis, of the telencephalon, just anterior to the nucleus entopeduncularis. In addition, intensely stained, widely distributed ir-granules, presumably representing the terminal fields of ir-NPY neurons, were found throughout the telencephalon (Fig. 15). These terminal fields were most abundant in the medial zone, the dorsal zone and the dorsal part of the lateral zone of the telencephalon. While ir-terminal fields were noted in all age groups, no ir-perikarya were noted in the telencephalon of animals from the initiation to the completion of puberty (ages 1.5 and 3 months).

The ventral tegmentum contained clusters of two to eight large perikarya that reacted strongly with anti-NPY. These perikarya were spherically-shaped with a single process that extended ventrally from the perikaryon. Immunoreactive perikarya in the ventral tegmentum were noted in all age groups studied.

Immunoreactive perikarya were also localized in the NPP. The appearance of ir-NPY in the NPP was not noted in neonatal and 3 months old animals.

Ir-NPY was localized in all three regions of the adenohypophysis as well as in the neurohypophysis (Figs. 3, 16). It did not appear to be exclusively associated with any one pituitary cell type. In the RPD immunoreactivity was noted in the

region of the gland known to be populated by prolactin-producing cells; in the PI it was noted in areas populated by cells that contain ir-GTH; in the CPD it was noted in areas populated by growth hormone-producing cells. The intensity of the immunoresponse to anti-NPY, in all age groups studied, was most pronounced in the PI and in the neurohypophysial tissue that invaded this region of the pituitary gland. This study did not establish the existence of ir-NPY and any pituitary hormone within the same cell.

Dynorphin

Ir-DYN was seen in the olfactory bulb and in the pituitary gland of all age groups studied. In the olfactory bulb, it was localized in granules surrounding cells in the ventral region of the bulb (Fig. 17). The relative amounts of immunoreactivity was significantly less in the olfactory bulb of neonatal animals than it was in older animals. Peak levels of the immune response were noted in pre-pubertal animals of 1 and 1.5 months of age.

In the pituitary gland, discrete clusters of tightly packed cells in the RPD and the PI were found to contain ir-DYN (Fig. 18). The clusters in the RPD comprised of two to six cells while those in the PI typically contained ten to twelve cells. Immunoreactivity in the RPD was confined to the region populated by prolactin cells and not to the adrenocorticotropin-producing cells of the pituitary. Immunoreactivity in the PI was confined to PAS+ cells that resided in areas of the gland that typically

are populated by cells that contain ir-GTH and not those associated with melanocyte stimulating hormone-producing cells.

Substance P

This study failed to find evidence of ir-SP in the brain or pituitary gland of platyfish at all ages.

Bradykinin

This study failed to find evidence of ir-BK in the brain or pituitary gland of platyfish at all ages.

Vasoactive Intestinal Peptide

This study failed to find evidence of ir-VIP in the brain or pituitary gland of platyfish at all ages.

II. Gonadotropin Releasing Hormones

The reader is referred to Table 5 and to Fig. 5 for a summary of the distribution of the variant forms of ir-GnRH.

Salmon GnRH

Immunoreactive (ir)- sGnRH was not seen in the brain of two day or 0.5 mos old animals. Ir-sGnRH was localized in perikarya of the NOR in animals aged 1.5 mos and older; there was no evidence of localization in neuronal processes in animals aged 1.5 mos. In animals aged 3 mos and older, ir-sGnRH was seen in both perikarya and axonal processes Figs. 5, 19). The ir-axonal processes were seen as beaded tracts that traveled one of two apparently different pathways. One pathway arched dorsally from the NOR and then extended caudally towards the NPP. Mammalian GnRH appeared to colocalized with sGnRH in this pathway. The other pathway extended along the ventral border of the brain, spanning the distance from the NOR to the NPP (Fig. 19, 20).

Mature animals (aged 3 mos and older) demonstrated ir-sGnRH in perikarya and fibers of the NPP (Fig. 21). The immunoreactivity appeared both in the cytoplasm of neuronal cell bodies and as ir-granules on the cell membrane of perikarya. Ir-fibers were also seen in the NLT. The processes in the NPP were directed in an anterior-posterior axis while those in the NLT had a dorsal-ventral orientation.

All animals aged 0.5 mos and older demonstrated ir-sGnRH in the CPD but the pattern of ir-cell distribution was markedly different among the different age groups studied. Animals aged 0.5 mos and younger had immunoreactivity only in the extreme lateral portions of the CPD (Fig. 22). Animals aged 1.5 mos demonstrated a response in the lateral portions of the CPD but also showed few, scattered cells in the ventromedial portions of this pituitary region. Animals aged 3 mos and older demonstrated an immunoreaction throughout the gonadotropic zone of the CPD (Fig. 23). Immunoreactivity to anti-sGnRH appeared to be around pituitary cells, either in the extracellular spaces between cells or in axonal processes and endings that innervate pituitary cells, as well as within the cytoplasm of pituitary cells.

Animals in all age groups studied had ir-sGnRH in the PI of the pituitary gland. The largest number of ir-cells were found in the center of this region (Fig. 22) and fewer were found in the lateral portions of the PI. In fact, lateral sections of the pars intermedia of animals of all ages, seldom showed any immunoreactivity to anti-sGnRH.

Ir-sGnRH colocalized with ir-GTH I in the PI of neonatal and immature animals. It was found to colocalize with both GTH I and II in older animals. This study did not determine any differential or preferential colocalization between ir-sGnRH and either ir-GTH I or ir-GTH II.

Mammalian GnRH

Ir-mGnRH was not observed in the brain of neonatal or 0.5 mos old animals. Animals aged 1.5 mos displayed immunoreactivity to anti-mGnRH in perikarya and processes of NOR neurons. Animals aged three mos and older demonstrated an immune response in perikarya and processes of the NOR as well as in processes of the NPP and NLT (Fig. 5). The processes in the three brain nuclei that responded to anti-mGnRH had the same orientation as those that responded to anti-sGnRH. The two antisera appeared to colocalized in fibers that seemed to exit the NOR, arch dorsally, then extend caudally through the NPO (Fig. 20). Fibers that demonstrated colocalization appeared thicker than those that stained with only one of the two antisera. Ir-mGnRH and -sGnRH were also colocalized within the cytoplasm of the cell bodies of NOR neurons.

In the pituitary gland, neonatal and 0.5 mos old animals had ir-mGnRH in cells in the extreme lateral regions of the CPD and in the PI (Fig. 24). The immunoreactive cells of the PI were arranged in a band that ran along the caudal end of the gland. This was similar to the arrangement of ir-sGnRH in the PI with one major exception: the ir-mGnRH cells decreased in number and became more scattered, losing the band-like arrangement, towards the center of the gland, where the majority of ir-sGnRH were found. Ir-mGnRH colocalized with ir-sGnRH in small clusters of cells in the center of the PI.

In animals aged 1.5 mos, the immune reaction to anti-mGnRH had increased in intensity in both the PI and the CPD when compared to younger fish. The band-like

arrangement of ir-cells in the PI was no longer observed. In the CPD, ir-cells were detected in a few scattered cells in the medial, ventral CPD as well as in the lateral sections.

At three months of age, ir-cells were found in large clusters in the PI as well as in the thick gonadotropic zone of the CPD (Fig. 25). In this age group, animals demonstrated a more intense reaction in the cells of the PI than in those of the CPD although there were some intensely stained CPD cells in some mature animals.

Colocalization of ir-mGnRH and -sGnRH was observed in both the CPD and in the PI. Ir-mGnRH also colocalized with ir-GTH I and II in a pattern that was similar to ir-sGnRH.

Chicken II GnRH

There was no response to anti-chIIGnRH in any animal under the age of 3 mos in either the brain or the pituitary gland.

In animals aged 3 mos and older, ir-chIIGnRH was localized in the NPP, NPO and in the NLT, but not in the NOR In the NPP, we observed sparse, scattered tracts that had the same orientation as tracts of ir-sGnRH containing neurons. In the NPO, ir-perikarya were observed anterior to the Habenula region and ir-axonal processes were seen as beaded tracts that existed either alone or in bundles (Fig. 26), ventral and caudal to the Habenula region. Beaded ir-fibers were also seen extending into the optic tectum, the ventral tegmentum and in the nucleus recessus lateralis (NRL) and posterioris (NRP) (Fig. 5). In the NLT, the distribution of ir-chIIGnRH fibers was

more concentrated in the posterior NLT where there was also an abundance of ir-granules that represented the terminal fields of ir-chIIGnRH-containing axons.

In the pituitary gland, the immune response to anti-chIIGnRH was observed both within the cytoplasm of pituitary cells in the CPD and as granules around the cells (Fig. 27).

In the PI, the immune response was considerably less intense than in the CPD, but there were occasional PI cells that demonstrated an intense reaction to this antisera.

We did not observe any colocalization of ir-chIIGnRH and other forms of ir-GnRH in brain nuclei even when ir-chIIGnRH was found in the same general brain regions as both ir-mGnRH and ir-sGnRH. There was a high degree of colocalization of ir-chIIGnRH and ir-sGnRH in pituitary gonadotropes in the CPD. Ir-chIIGnRH was observed around pituitary cells that reacted to anti-GTH I and II as well as colocalized with ir-GTH I and II within the cytoplasm.

Lamprey GnRH

Ir-lGnRH was not seen in any brain center of any animal studied. Immunoreactivity was restricted to the pituitary gland in all age groups. Very intense reactions were observed in the PI in all age groups. In animals aged 0.5 mos and younger, the immunoreaction was only seen in clusters of PI cells (Fig. 28). In animals aged 1.5 mos, an immune response in the ventral CPD was observed. At, this age, the response was only demonstrated by relatively small number of cells compared to the response in the PI. While the amount of ir-material in the CPD increased in

animals aged three months and older, it never encompassed the entire ventral CPD zone of gonadotropes (Figs. 29). Its distribution in the gonadotropic zone of the CPD was considerably more limited, while its distribution in the PI was as prominent as, any of the other ir-GnRH forms studied here.

The reaction to anti-lGnRH appeared to be fairly restricted to the cytoplasm of pituitary cells with very little ir-material in the extracellular spaces.

In the PI of neonatal and 0.5 mos old animals, ir-lGnRH colocalized with ir-mGnRH in some cells that formed the band of ir-mGnRH-containing cells described above. In mature animals, ir-sGnRH, -mGnRH, and -chIIGnRH were localized around cells containing ir-lGnRH, and in the case of ir-mGnRH and -sGnRH, there was also some colocalization with ir-lGnRH within cells. Cells that contained ir-lGnRH appeared to contain ir-GTH I and/or II, as well.

III. Gonadotropins I and II

Neonatal animals (2 days) demonstrated immunoreactivity to anti-GTH I in the cells of the PI of the pituitary gland. In animals aged 0.5 mos, the immune reaction was seen in both the PI and in the lateral sections of the CPD. This immunoreactivity increased in animals aged 1.5 mos (this age marks the initial stages of puberty in these platyfish stocks) to include a thin band of cells that spanned the ventral border of the CPD (Fig. 30). The staining in the cells of the CPD was consistently more intense that in the PI. By three months of age (the process of sexual development is nearly

completed and animals are young adults), all animals demonstrated a response to anti-GTH I in a thick band of gonadotropes in the ventral CPD and in the PI (Fig. 31).

There was no response to anti-GTH II in neonatal and 0.5 mos old animals. At 1.5 mos of age, animals demonstrated a response to this antisera in a thin band of cells along the ventral border of the CPD (Fig. 32). By three months of age, animals demonstrated immunoreactivity in the thick band of gonadotropes in the ventral CPD and in scattered clusters of cells in the PI (Fig. 33).

In three month old animals, there was a significant degree of colocalization of anti-GTH I and anti-GTH II in both the PI and the CPD. However, in both regions of the pituitary gland, there were also cells that stained exclusively with one or the other of the two antisera.

IV. Amino Acid Neurotransmitters

This study failed to find immunoreactivity to antisera to GABA or GLU in the brain or pituitary gland of platyfish at any age studied.

V. Serotonin

Neonatal animals demonstrated ir-serotonin containing perikarya in the NPP, the nucleus recessus lateralis (NRL), the nucleus recessus posterioris (NRP), the valvula of the cerebellum (VC) and the area of the third ventricle, near the nucleus ventromedialis thalami (NVT). These perikarya appeared to be ovoid, unipolar neurons. There were no detectable ir-fibers in this age group (Fig. 37).

In animals aged 0.5 mos and older, ir-perikarya were localized in all the same regions as in the younger fish. In addition, ir-neurons were found in the VT and ir-cells seen in the pineal gland. Immunoreactive fibers, in animals aged 0.5 mos and older, were seen scattered throughout the NPO with a rostrocaudal orientation. Ir-fibers were also seen in the lateral NPP, were they had the same orientation as in the NPO, and in the NLT. In the NLT, these fibers did not have a definitive orientation.

In the pituitary gland, immunoreactivity to anti-serotonin was first noted in neonatal animals in the cells of the PI. At this age, there was also a pale immune response in cells of the RPD. In animals aged 1.5 mos and older, immunoreactive pituitary cells were seen in small clusters in the CPD as well as in the PI. The immune response in the cells of the RPD was still very pale compared to that in the PI cells.

VI. Dopamine

This study utilized two different antibodies, a glutaraldehyde-conjugated antibody (DA1) that was used on both Bouin's-fixed and glutaraldehyde-fixed tissues, and a formaldehyde-conjugated antibody (DA2) that was used only on Bouin's-fixed tissues. The immune response to DA1 was identical in both glutaraldehyde- and Bouin's-fixed tissues.

Neonatal and 0.5 mos old animals did not demonstrate an immune response to either antisera. With DA2, all animals aged 1.5 mos and older had scattered, short ir-fibers with no distinctive orientation in the medial telencephalon, and the NPO. More

prominent, longer, thicker, ir-fibers with a dorsoventral orientation, were seen in the NRL and the anterior and ventral NLT. When antisera to DA1 was used, no immunoreactivity was noted in the telencephalon and the immune response in the NRL and NLT was considerably paler than that with DA2. Increasing the concentration of the DA1 antisera in the final dilution only served to heighten background staining but did not increase the specific staining in the NRL and NLT.

In the pituitary gland, the difference in the immune response to the two antisera was marked. Neonatal and 0.5 mos old animals demonstrated an immune response to DA2 in the cells of the CPD and the RPD. In animals aged 0.5 mos, anti-sera to DA2 appeared to colocalize with anti-sera to GH in the cells of the CPD. In animals aged 1.5 mos and older, anti-sera to anti-DA2 seemed to colocalize with anti-GTH in the ventral CPD. There was no colocalization noted between ir-DA2 and ir-GH in animals aged 1.5 mos and older.

The immune response to anti-DA2 remained uniform in all age groups.

In sections treated with anti-DA1, no CPD staining was noted in any age group studied. All animals, in all age groups, demonstrated an intense immune response to this antisera in the cells of the RPD which colocalized with an equally intense response to anti-prolactin.

VII. Steroid Receptors

Only male platyfish were utilized in this study except for neonatal animals whose sex could not yet be determined by either body pigment pattern, anal fin differentiation or histological examination of the gonad.

Animals under the age of 1.5 mos did not demonstrate immunoreactivity to antisera to androgen receptors in the brain. At 1.5 mos and older, males demonstrated an immune response in the neurons of the ventral NLT, pars inferioris (Figs. 34, 35). This response was limited to the nucleus of these cells.

Animals of all ages demonstrated an immune response to this antiserum in the pituitary gland. The response was limited to cells of the PI in neonatal and 0.5 mos old animals. In animals aged 1.5 mos and older the response was seen in cells of the ventral CPD and at the boundary between the RPD and CPD as well as in the cells of the PI (Fig 36). The response was noted in the cytoplasm as well as in the nuclei of reactive cells.

VIII. Exogenous Administration of Neuropeptide Y

Immature animals

Animals receiving injections of NPY demonstrated a greater percent increase in their standard length than did control animals for the first three weeks of injection therapy (Fig. 38). In the final two weeks of the experiment, control animals demonstrated a greater percent increase than did animals receiving the hormone. At

the end of five weeks, there was no statistical difference in the percent increase in standard length between the two groups.

Animals receiving NPY injections demonstrated a statistically significant increase in the number of cells that were immunoresponsive to antisera to GH than did animals receiving saline (Fig. 39).

There were no detectable differences in the development of the gonadotropic zone in the ventral CPD or in the number of cells immunoresponsive to antisera to GTH between the two groups.

There was no discernible difference in the rate of gonopodial development between animals receiving NPY and those receiving saline.

Histological analysis of the gonads also showed no discernible difference in stage of maturation between the two groups.

Mature animals

Daily NPY injections appeared not to have any effect on mature animals (Figs. 38, 39). There were no discernible differences in the number of cells immunoresponsive to either GTH or GH. There were no differences in gonadal structure and no statistical differences in percent increase in standard length.

Discussion

The genus *Xiphophorus* provides valuable research models for the study of the genetic and neuroendocrine influences on reproductive system development, maturation and function (cf. Schreibman and Margolis-Nunno, 1987, 1989, Schreibman, *et al.*, 1994). Additionally, the regional distribution of pituitary cells according to physiological function, and their direct hypothalamic innervation, makes the teleost pituitary an excellent model in which to investigate the interaction between the central nervous system and the pituitary gland (cf. Schreibman *et al.*, 1973; Schreibman, 1986).

The NOR is believed to be the site of the initiation of a cascade of neural signals that descends upon the pituitary gland resulting in the secretion of pituitary gonadotropin and the subsequent maturation of the gonads (Schreibman *et al.*, 1982). The NPP and the NLT, are believed to function in modulating this cascade and thus perfecting the timing of the onset of puberty. In light of these facts, it is interesting that ir-FMRF-amide and ir-GnRH colocalize in NOR neurons. These two peptides do not coincide in all perikarya of the NOR suggesting that the NOR is not a homogeneous cluster of cell bodies but rather is comprised of subpopulations of morphologically similar neurons that are physiologically different (a division of labor?). This interpretation is substantiated by our finding that ir-NPY is seen in only a limited number of NOR perikarya.

The colocalization between the ir-FMRF-amide and -GnRH was also noted in the neural network of fibers in the *Xiphophorus* brain. While colocalization in fibers

cannot be definitively demonstrated by immunocytochemistry at the light microscope level, there appears to exist several anatomically and functionally distinct systems, one of which contains both FMRF-amide and GnRH. Batten and associates (1990) reported similar results in the green molly, *Poecilia latipinna*. They found that while there was extensive colocalization of ir-GnRH and -FMRF-amide in NOR neurons and in neural tracts, numerous fibers stained solely with antisera to FMRF-amide and some solely with GnRH.

In the absence of a median eminence, the teleost adenohypophysis is regulated by hypothalamic neurons that terminate either directly on adenohypophysial cells, in the extracellular spaces between adenohypophysial cells or on the basement membrane that separates the neurohypophysis from adenohypophysis. Many investigators believe that the NLT is a source of adenohypophysial-regulating neurons (Peter and Fryer, 1983). Therefore, the presence of ir-FMRF-amide in the NLT is reason to suspect this peptide of having a role in pituitary regulation via an effect on the neurons of the NLT. The absence of significant levels of ir-FMRF-amide in the pituitary gland would argue against a direct effect of FMRF-amide on this gland. However, the prominence of ir-FMRF-amide in two GnRH-containing brain nuclei, one of which is the NLT, with increased arborization of ir-FMRF-amide-containing fibers coinciding with the onset of sexual maturity, suggests that this peptide is involved in pituitary regulation. This involvement could consist of an effect on GnRH and/or GnRH activity via an effect on NLT and/or NOR neurons. Rama Krishna *et al.* (1992), reported that fibers containing ir-FMRF-amide arose from nervus terminalis neurons and directly

innervated adenohypophysial cells in the catfish, *Clarias batrachus*. Chiba *et al.* (1991), reported, in the cloudy dogfish, *Scyliorhinus torazame*, some ir-FMRF-amide labeled fibers terminated around the vascular wall of the primary capillary plexus of the median eminence while others penetrated the adenohypophysis. This evidence strongly suggests that FMRF-amide-like peptides are involved in pituitary regulation.

The importance of the NLT in pituitary functions involving reproduction is underscored by our finding ir-androgen receptors in this brain nucleus as well as in the pituitary gland. The absence of such receptors in other nuclei would seem to indicate that the NLT is a prime target of gonadal feedback control on the pituitary gland. However, this report has also established that there is feedback control aimed directly at pituitary cells and that this direct control is not exclusively exerted on pituitary gonadotropes. We found ir-androgen receptors on cells at the RPD/CPD boundary, an area populated by corticotropes, indicating that these cells may also be influenced by circulating levels of gonadal steroids.

Likewise, the localization of ir-GAL in all three regions of the adenohypophysis, suggests that this peptide directly affects more than one aspect of pituitary gland function in *Xiphophorus*. Our observation that ir-GAL and -growth hormone occur concurrently in pituitary somatotropes of the CPD suggests a correlation between these two peptides at the cellular level. GAL may also influence growth hormone secretion via an effect on somatotropin neurons as suggested in mammals (Maiter *et al.*, 1990; Hulting *et al.*, 1991; Tanoh *et al.*, 1991). In fish, Batten (1990) demonstrated PAP-labeled ir-GAL fibers contacting growth hormone

cells in the sea bass, *Dicentrarchus labrax*, pituitary at the ultrastructure level, but a later study by Moons *et al.* (1991), on the same species, failed to demonstrate GAL binding sites on growth hormone cells by autoradiographic studies. These observations appear to be contradictory but they are not mutually exclusive. Mismatches between immunocytochemical peptide localizations and receptor studies are not uncommon and indeed, the observations may have physiological relevance. Given the evidence of an anatomical link between GAL and growth hormone, the relationship between these two peptides needs to be further studied on a functional level to fully determine the effect of GAL on growth hormone secretion and the mechanism by which such an effect is exerted.

In mammals, GAL colocalizes with arginine vasopressin (AVP) in neurons of the supraoptic and paraventricular nucleus (Brownstein and Mezey, 1986; Melander *et al.*, 1986; Rokaeus *et al.*, 1988; Gayman and Martin, 1989; Skofitsch *et al.*, 1989). The NPO of the teleost is homologous to the paraventricular/supraoptic nuclei of mammals (Crosby and Showers, 1969). While both ir-GAL and ir-arginine vasotocin (AVT), the fish counterpart to AVP (see Holmqvist and Ekstrom, 1991), were localized in NPO perikarya and fibers in this study, they were not found to coexist in the same neurons. This agrees with reports by Holmqvist and Ekstrom (1991) in the Atlantic salmon, except that they found ir-GAL perikarya in the nucleus recessus lateralis and in the nucleus recessus posterioris, while we did not.

This study did not discover many major differences in the distribution of the three ir-peptides among the three stocks of *Xiphophorus* included in this study. There

appears to be an interesting pattern of age-related differences in the distribution of ir-GAL and -NT among the three stocks. It seems that the earliest maturing stocks, the marigold wagtails, were found to possess ir-GAL and -NT in the PI at two days of age, several weeks before the other two stocks.

The PI of neonatal *Xiphophorus* is of interest in development because it is known to contain, among its other cell types, a class of cells that contain ir-GTH I, a form of gonadotropin hormone believed to be associated with early sexual development and the maintenance of the immature gonad (Schreibman *et al.*, 1990; Magliulo-Cepriano, *et al.*, 1994)). In addition, PI cells contain ir-Somatolactin (SL) (unpublished, with M. Rand-Weaver). Somatolactin is a newly isolated hormone whose function is not yet known, but which is reported to be involved in sexual maturation and development of fish (Planas *et al.*, 1992; Rand-Weaver *et al.*, 1992; Rand-Weaver and Swanson, 1993) and in reproductive function (Olivereau and Rand-Weaver, 1994). Our study has determined that ir-GAL colocalized with ir-SL in the PI of *Xiphophorus*. While an anatomical identity between substances or between cell types does not necessarily indicate a functional link, the prevalence and nature of structure-function relationships in the biological world is such that it renders such correlations worthy of further investigation. Physiological studies assaying for GAL and NT in animals undergoing development might elucidate a role in the developmental process for these peptides.

This study did not find as extensive a system of ir-NT fibers in the brain as reported by Batten *et al.*, (1987). They reported fibers in the NPO, NPP, in the

nucleus anterioris periventricularis (NAP), as well as in the lateral and posterior NLT. We found fibers and an extensive terminal field in the anterior NLT. This could be due to species differences, for although Batten studied several species of teleosts, none were of the genus *Xiphophorus*. Differences in the age, sex and physiological states of the animals studied could also provide for significant variation in distribution results. Variations in antisera and antisera affinity for the antigen may also explain the differences observed. The presence of ir-NT in the NLT strengthens the possibility of a role for this peptide in pituitary function.

Our results in the pituitary gland, however, closely parallel those of Batten and his associates in that ir-NT was localized in the neurohypophysis bordering the ACTH cells and also in close proximity to prolactin cells. We agree with Batten that these findings raise the possibility that NT may modify the functioning of the ACTH cells and perhaps the nearby prolactin cells as well. It is has been demonstrated by Schreibman and co-workers that the olfactory system of platyfish undergoes dramatic changes at the time of sexual maturation and coincident with the appearance of ir-GnRH in the olfactory bulb (Schreibman, *et al.*, 1984). In pre-pubescent platyfish the olfactory epithelium consists of a mass of poorly vascularized, uniform, undifferentiated cells. As the animal approaches and proceeds through puberty, dramatic changes occur in the olfactory epithelium transforming it into a highly vascularized structure populated by specialized cell types (Schreibman, *et al.*, 1984).

The association between the olfactory system and GnRH neural networks is well-documented. GnRH neurons have their embryonic origins in the olfactory

placode. During development, these neurons migrate from the placode to their destination in various brain nuclei. An inherited disorder in humans known as Kallmann's syndrome, results in, among other things, hypogonadism and anosmia. The syndrome is caused by the failure of GnRH neurons to leave the olfactory placode and embark on their migratory adventure (Schwanzel-Fukuda, *et al.*, 1992). Stuck in the placode, the GnRH neurons cannot fulfill their intended role, and subsequently, the gonads do not develop properly. It is not certain whether the anosmia is the result of insufficient GnRH levels in appropriate areas or whether it is due to a lack of feedback from other GnRH-dependent structures, but the connection between GnRH and the olfactory placode is intriguing. In addition, light and electron microscope studies have established that neural processes from olfactory cell processes synapse with the cells of the NOR (cf. Schreibman and Margolis-Nunno 1987).

Thus, our demonstration of ir-DYN in both the olfactory bulb and in the pituitary gland is of interest in light of these structural and functional links between the olfactory system and neuroendocrine structures connected to reproduction and the suggested role of this peptide in reproductive physiology (Kalra and Kalra 1991). It is also of special interest that ir-DYN was localized in cells of the PI that correspond to those that are PAS+ and immunoreactive to anti-GTH. These cells, unlike the gonadotropes of the ventral caudal pars distalis, are present at birth and are immunoreactive to antisera to one form of coho salmon gonadotropin, GTH I, but not to a second form, GTH II (Schreibman *et al.* 1990). The presence of ir-DYN in pituitary cells that have been associated with GTH I, together with its presence in the

olfactory bulb, makes this opioid an interesting candidate for further studies into the neuroendocrine aspects of sexual development in teleosts.

The presence of NPY in the NOR is of prime interest. This study did not detect any colocalization between ir-NPY and ir-FMRF-amide or between ir-NPY and ir-GnRH. Our observation that NPY immunoreactivity occurred in only four to twelve of the approximately 80 perikarya that make up this nucleus (Halpern-Sebold *et al.* 1986), indicates that this nucleus is a heterogenous mix of morphologically similar but physiologically different neurons. This needs to be further explored to resolve questions of colocalization of products and the possibility of a division of labor and/or a specialization of function among NOR neurons, as we suggest.

Neural processes projecting from ir-NPY containing NOR neurons extend dorso-posteriorly towards the NPO and the NPP indicating that there may be communication among these nuclei. It has been suggested that the preoptic area is a site of the inhibition of GnRH release by dopamine in goldfish (Peter *et al.* 1990) and the NPP has been proposed as a site for gonadal steroid-feedback action (Demski, 1984). This study did not discover ir-steroid receptors in the NPP and yet the role of this nucleus as a "brake", as somehow having an inhibitory influence on GnRH secretion, has been hypothesized in platyfish (Schreibman, *et al.*, 1990). It is, therefore, interesting to note that ir-NPY was not localized in the NPP of neonatal animals, in whom the sexual development process had not yet begun, and in three month old animals, in whom the process had reached completion, but was present in all other intermediary age groups examined. Perhaps, the inhibitory influence of this

nucleus is mediated, not by negative feedback via steroids, but by NPY activity in this nucleus.

The developmental, stage-related changes in the presence of ir-NPY in the telencephalon (absent at 1.5 and 3 months of age) may suggest a role for this brain region in the maturational process. This is thought provoking in light of the fact that other reports have indicated the presence of ir-GnRH-containing perikarya and fibers in the telencephalon of teleosts (Goos and Muranthanoglu, 1976; Demski, 1984). In studies of three other teleosts (goldfish, Pontet *et al.* 1989; green molly, Batten *et al.* 1990; trout, Danger *et al.* 1991), ir-NPY was localized in the pituitary gland and in the telencephalon. Batten and associates found ir-NPY fibers extending through the dorsal telencephalon of the molly brain, a region in which we found the terminal fields of ir-NPY neurons. The telencephalon is also known to contain steroid-concentrating neurons (Demski, 1984) and several investigators believe that the telencephalon is involved in reproductive behavior and gonad function, although others dispute this hypothesis (Davis and Kassel, 1984). These finding provide tantalizing, if still tenuous, links between NPY and GnRH activity in the telencephalon. We must be cautioned, however, that NPY is a neurotransmitter that may modulate many functions and behaviors, some of which are likely to be regulated by the neurons of the telencephalon.

Evidence in mammals suggests that NPY influences GTH secretion via its influence on GnRH neurons (Tillet, *et al.*, 1989; Kaynard, *et al.*, 1990; Sahu, *et al.*, 1990; Parker, *et al.*, 1991; Woller and Terasawa, 1991). The presence of ir-NPY in

ir-GnRH containing brain nuclei, but not in the ventral CPD gonadotropes, of the platyfish and other teleosts, would support this theory.

Among the teleosts discussed above (trout, goldfish and molly), the platyfish was the only animal in which ir-NPY was localized in the NOR. However, due to the experimental design of those studies, this nucleus was not examined in the other three species. What is clear and consistent, is that NPY, by virtue of its distribution in select centers of the brain and in the pituitary gland of teleosts, is a major candidate for a regulator of pituitary and neurophysiological function.

In vitro studies have demonstrated that NPY stimulated GTH secretion from perifused goldfish pituitaries (Peng *et al.* 1990). The conspicuous absence of ir-NPY in the ventral zone of the caudal pars distalis of the platyfish pituitary, along with the results of our NPY injection experiment, would argue against a direct effect of this peptide on pituitary gonadotropes in this animal. NPY, like FMRF-amide, would be more likely to affect pituitary gonadotropes by influencing the activity of GnRH neurons since this peptide has been localized in close proximity to GnRH neurons. The presence of ir-NPY and ir-FMRF-amide in the neurohypophysis could have several implications, particularly in light of the fact that teleosts lack a median eminence. Besides the fact that adenohypophysial cells are regulated by direct innervation of hypothalamic neurons, some of which penetrate the adenohypophysis and by others which terminate in the neurohypophysis, pituitary cells can also be influenced by neuroendocrine factors that percolate through the gland. Granules of ir-NPY in the neurohypophysis could affect several pituitary cell types by filtering

into the adenohypophysis or they could effect the axonal terminals located in the neurohypophysis itself. Since ir-NPY was not seen in the ventral caudal pars distalis, where the gonadotropes are located, but only localized in the neurohypophysis, it does not seem likely that NPY could affect the gonadotropes directly. However, the possibility does exist that NPY influences the axonal terminals of GnRH-containing neurons in the neurohypophysis.

An *in vivo* study conducted by Breton, *et al.* (1991), in rainbow trout and common carp concluded that NPY injections stimulated GTH release. However, their report failed to establish whether this was due to activity at the pituitary level or by activation of central mediatory systems, as we suggest.

The absence of direct NPY activity at the level of pituitary gonadotropes was confirmed by our injection study. Intraperitoneal injections of this hormone did not alter gonadotrope development or morphology. It has been suggested by other studies that this hormone may act to potentiate the action of GnRH on GTH cells (Crowley, *et al.*, 1987; Sedan, *et al.*, 1988). However, based on our study, it now seems probable that any effect that NPY may have on pituitary gonadotropes, either in development or in the functioning of the mature reproductive system, is negligible.

Our findings that administration of exogenous NPY results in the proliferation of pituitary somatotropes confirms previous studies that have indicated that NPY is involved in pituitary growth hormone physiology in mammals (Scabbed, *et al.*, 1988; McDonald, *et al.*, 1985) and in fish (Peng, *et al.*, 1990). Based on our findings, it seems that NPY may have a direct and profound effect on somatotrope development

and proliferation and, furthermore, that this effect is a time-locked event occurring at a restricted time during development.

These findings may indicate that NPY is a major regulator of peripheral, but important, aspects of sexual development, such as size. In platyfish, the *P* gene is believed to control both the age and size at which sexual maturity will occur. In another species of *Xiphophorus, X. multilineatus*, *P* genotypes are more clearly correlated with size at maturity rather than with age (Kallman, 1989; Schreibman, *et al.*, 1994, 1990). By changing the conditions under which these animals are reared, one can modulate the age at which maturity will occur, but not the size.

In vitro studies conducted by Peng, *et al.* (1990) indicated that NPY stimulated both GH and GTH release in perifused goldfish pituitary gland. This would appear to contradict some of our findings. However, it must be remembered that both *in vitro* experiments and injection of pharmacological doses of a hormone, are artificial situations that are capable of shedding light only on what may possibly happen *in vivo.* The behavior of a pituitary gland in a perifusion system may be very different from that of the same pituitary in its natural milieu and it may also be that physiological doses of a hormone have one effect while pharmacological doses have the opposite effect.

The finding that exogenous NPY can effect somatotrope proliferation may have practical value. Aquaculture, or fish farming, is an ever-growing industry. One object in the culture of commercially valuable organisms is not, necessarily, to grow bigger fish, but rather to grow fish that will reach their maximum or marketable size in less

time. On the basis of the findings of this study, it would seem that is exactly what exogenous administration of NPY accomplished. At the end of five weeks, the NPY-injected fish were not bigger than the controls, but they had reached their present size faster than the controls and this was most likely due to the significant proliferation of GH-producing cells noted in the pituitary gland of animals injected with NPY.

The morphological, anatomical and physiological findings of this study further support the notion of FMRF-amide, GAL, NT, NPY and DYN involvement in pituitary gland functions related to growth and reproduction. Furthermore, NPY, DYN, NT, and GAL were peptides that were originally isolated and characterized in mammals. The chemical identities of the ir-peptides found in teleosts, and specifically in *Xiphophorus*, remains to be elucidated by extraction, isolation and characterization, however, they would appear to be closely chemically related to, and perhaps even identical to those peptides characterized in mammals. This would indicate a high degree of structural, and perhaps functional, conservation of these peptides throughout vertebrate evolution thus lending further credence to their importance as physiological regulators in vertebrate systems.

The results of our study with variant forms of GTH and GNRH demonstrate that multiple forms of GnRH and GTH are present at defined stages of *Xiphophorus* development and that they are associated with specific morphological regions of the brain and pituitary gland (Table IV). The findings may also suggest that different forms of GnRH and GTH are associated with different aspects of reproductive system development and function. The pattern of localization of ir-GTH I in the various age

groups, parallels the sequence of development of pituitary gonadotropes from early immature stages to sexual maturity. Ir-GTH II, in contrast, does not appear in immature stages, but rather, makes its debut in the initial stages of puberty. These findings are consistent with those in other teleost species in which GTH I was seen, by immunocytochemistry, chronologically prior to GTH II (Naito *et al.*, 1988). The differential appearance of the two gonadotropins argues for differential functions for the two and this also is consistent with studies that indicate different physiological roles for these two peptides (Suzuki *et al.*, 1988a; Swanson *et al.*, 1989; Kawauchi *et al.*, 1989).

The pattern of the appearance of ir-sGnRH and ir-mGnRH, in the various age groups, is also highly significant in light of our knowledge of the physiology of development. In the brain, these peptides are already seen in the NOR, at 1.5 mos of age, followed by their appearance in the NPP and the NLT. This pattern of sequential developmental neural events is believed to result in the onset of puberty. The sequence of the appearance of these ir-peptides in the pituitary gland, parallels the developmental sequence of the gonadotropic zone. This suggests a role for these peptides in events associated with the neuroendocrine regulation of the onset of puberty as well as with early gonadotrope physiology. Further investigation is required to determine what differential functions these two ir-peptides serve in these events.

Similarity in the structure of antigens may raise issues of concern in the localization of these antigens by immunocytochemistry. Even more problematic may be determining colocalization or coexistence of similar antigens within the same cell

or structure since color evaluation can present difficulties. The results of this and all such investigations must be evaluated in light of these considerations. In our studies, we sought to circumvent and minimize the problems associated with structure similarity and color evaluation by utilizing pre-absorbed antisera when necessary, following stringent methods and controls, and for our colocalization, using chromogens that would yield clear, distinctive results. However the possibility of cross reactivity with non-specific molecular entities can not be absolutely ruled out.

Detailed HPLC and RIA studies have not, as yet, involved the members of the genus *Xiphophorus*. Preliminary HPLC studies in platyfish have indicated the presence of the mammalian, chicken II and salmon varieties (Schreibman and Sower, unpublished). Lamprey GnRH was not found but these studies did not include the pituitary gland where we have now localized ir-lGnRH.

The restriction of ir-lGnRH to the pituitary gland is puzzling. The inability to localize lGnRH in the brain would seem to indicate that it does not have a major hypophysiotropic role. However, this study demonstrated a very intense response to anti-lGnRH in pituitary gonadotropes at all ages. This may indicate that lGnRH is synthesized in the brain in amounts too minute to detect by immunocytochemistry, and is then pooled in the pituitary. Another explanation, although not a currently popular view, could be that lGnRH is synthesized in the pituitary gland itself and may perhaps, serve a paracrine or autocrine role in pituitary regulation.

This is the first report of an lGnRH-like molecule found in teleosts. This form of GnRH was believed to be restricted to agnathans. However, given the results of our

absorption study and the distinctness of the distribution of immunoreactivity, this report should rekindle interest and spur further investigations into the phylogenetic distribution of lGnRH.

In 1987, Idler and Everard demonstrated by chromatographic mobility and immunoreactivity, the presence of mammalian-, salmon- and chicken-like LHRH molecules in the hypothalamus of the winter flounder, *Pseudopleuronectes americanus*. These results essentially parallel our own. Barnett and co-workers (1982) also demonstrated three forms of GnRH-like molecules in the brain of a teleost, but they believed that none of the three were mammalian-like. Species differences may account for differing results among the teleosts, but, all in all, it now seems highly probable that two or three or more variant GnRH molecules can exist in a single teleostean species.

The absence of ir-chIIGnRH in the brain and pituitary gland of developing animals and its sudden appearance in sexually mature animals at 3 mos of age is one of the more interesting phenomena uncovered by this study. It would appear that chIIGnRH is involved in orchestrating events leading to the completion of puberty, successful mating, and reproduction, but is not involved in the early developmental events. That a division of labor exists between chII and other forms of GnRH is supported by our finding that ir-chIIGnRH did not colocalize with other forms of GnRH in the brain, although it was found in the same general regions. Ir-chIIGnRH only colocalized in the pituitary with ir-GTH I and II, and with ir-sGnRH in gonadotropes. It was seen with other ir-GnRHs surrounding gonadotropes in mature

fish. This implicates chIIGnRH in pituitary gonadotrope function yet establishes a physiological distinction between chII and other forms of GnRH.

Ir-chIIGnRH was also the only one of the four ir-GnRHs to be seen in the midbrain. This is consistent with other reports that have indicated that there may be anatomically distinct GnRH systems in the teleost brain, utilizing variant forms of the decapeptide (Yu, *et al.*, 1988; Batten, *et al.*, 1990) and suggesting that distinct systems regulate different functions (Sherwood, *et al.*, 1993). Among teleosts in general, there appear to be three significant systems of GnRH-containing brain neurons, namely, the NOR (or the terminal nerve, as it is known in some species), the pre-optic region and the midbrain (Munz, *et al.*, 1981; Kah, *et al.*, 1986; Subhedar and Rama Krishna, 1988; Batten, *et al.*, 1990; Amano, *et al.*, 1991; Grober and Bass, 1991). Studies on the presence of GnRH in the telencephalon and the diencephalon far out-number those concerning GnRH in the midbrain even though GnRH has been reported in the midbrain of birds (Mikami, *et al.*, 1988), reptiles (Bennis, *et al.*, 1989), and fishes (Munz, *et al.*, 1981; Borg, *et al.*, 1982; Miller and Kriebel, 1986; Wright and Demski, 1991). It has been suggested that the existence of this midbrain GnRH system promotes not only the likelihood of GnRH having other, non-hypophysiotropic, roles but may also provide an anatomical basis for physiological studies that have suggested that GnRH directly regulates or facilitates sexual behavior (cf. Sherwood, *et al.*, 1993).

Future studies in this field must include an investigation of ages and stages of development intermediate to those studied here. The precise timing of the appearance

of ir-chIIGnRH must be determined so as to further understand the relationship of this hormone to GTH II and the development process. Furthermore, earlier studies determined that ir- mGnRH appears in the NOR at 5 weeks of age (Schreibman and Margolis-Nunno, 1987). It must be determined if ir-sGnRH is also present at an earlier age that the 1.5 mos age group examined here.

Reports have suggested that one or more variant forms of GnRH, occurring in a single species, may function as neurotransmitters (Yu *et al.*, 1991; Okuzawa *et al.*, 1990). The findings of this study indicate that in the genus *Xiphophorus*, a role for any one of the variant forms of GnRH investigated here, as neurotransmitters, would exist in addition to its primary role as a hypophysiotropic factor. Three of the four forms of ir-GnRH studied were found in brain regions previously implicated (Schreibman and Margolis-Nunno, 1987, 1989) in reproductive system physiology; all were found in the pituitary gland; all were found to be associated with gonadotropes. This evidence strongly suggests an important hypophysiotropic function for these peptides.

GnRH is believed to serve multiple functions, even in the pituitary gland where it as been shown to have growth hormone releasing properties in goldfish (Marchant, *et al.*, 1989). In rats, 30-50% of the axons of neurons of the septal-preoptic-hypothalamic region are distributed throughout the brain rather than going to the median eminence (Silverman, *et al.*, 1987; Merchenthaler, 1991). In this study, we report ir-chIIGnRH tracts in the optic tectum and in the ventral tegmentum as well as in brain regions we believe to be associated with reproduction. It is therefore very

probable for a single form of GnRH to have dual or perhaps multiple functions within a single species.

This report also raises the question of the functional significance of variant GnRH forms at the cellular level. Given the limitations of the methods, the evidence presented here strongly indicates that there is colocalization among the variant forms of ir-GnRH. It would be interesting to determine the specific interaction between these variant forms and pituitary cells or brain neurons. There have been other reports on the coexistence of two GnRH variants being released from a single neuron and binding to the same receptor (see Sherwood, *et al.* 1993). Given the colocalizations observed here, it is likely that the various forms of GnRH are performing different intracellular functions, perhaps through different post receptor pathways (Chang, *et al.*, 1993; Levavi-Sivan and Yaron, 1992; 1993). However, this can not be determined without extensive physiological, biochemical and molecular manipulation and experimentation. Based on the specific sites of their localization, it is likely that both salmon and chicken II GnRH are required for endocrine activity associated with reproduction. The localization of ir-GnRH inside and outside of cells may reflect different phases of reproductive system development, varying levels of circulating hormones and the availability and activity of hormone receptors.

Investigations concerning the GnRH family of peptides have relied most heavily on protein chemistry and chromatographic and immunological data. While the gene for mammalian luteinizing hormone-releasing hormone was first characterized by Seeburg and Adelman in 1984, it is only very recently that the cDNA structure of

GnRH variants in fish have been reported (Bond, *et al.*, 1991; Coe, *et al.*, 1992; Grober, *et al.*, 1992; Klungland, *et al.*, 1992). It will be very interesting to see how future molecular data impacts on data already generated in this field. Although different methods of investigation capture different aspects of a very dynamic cycle, *in situ* localization of GnRH genes and mRNAs will be an important facet of the overall understanding of the GnRH peptides and their functions.

Ir-GABA and -GLU were not found in the brain or pituitary gland of platyfish. Given the ubiquitousness of these amino acids in other species, the failure to localize these antigens was most probably due to technical problems associated with the detection method and with the antibody itself. This study should be repeated, perhaps with antisera from a different source and employing a variety of fixation methods. Kah and co-workers (1987) in an electron microscopic study involving pre-embedding immunocytochemistry, proved successful in localizing immunoreactive GABA neurons in the brain of the goldfish. In this study, immunocytochemistry was conducted on polyfin-embedded tissue and utilized a 3% glutaraldehyde fixative on the premise that the antibody had been generated against a GABA-, or GLU-glutaraldehyde conjugate and would therefore only recognize GABA that had been fixed by glutaraldehyde. As the results with the two antisera to dopamine demonstrated, this is not always the case. Perhaps, as newer, improved antisera becomes available, and different processing techniques are developed and refined, the localization of these amino acid neurotransmitters in the teleost brain will help

illuminate the role these amino acids play in the regulation of reproduction in *Xiphophorus.*

The results of our serotonin study essentially paralleled those of an earlier report by Margolis-Kazan and co-workers in 1985 and extended the available information to include younger animals. Serotonin appears to have a fairly wide distribution in the platyfish brain at a very early stage of development and this distribution was uniform throughout the age groups examined here. This would lend greater importance to the immunocytochemical changes, reported by Margolis-Nunno and colleagues (1986) in older, aging platyfish and bolster the suggestion that these changes are associated with reproductive senescence.

Our dopamine study, utilizing two different antisera to the same antigen, provided us with some interesting results and some puzzling ones as well. Obviously, the two antisera had differing affinities, but theoretically speaking, and according to the supplier's information and to standard practice, the DA1 (antisera to glutaraldehyde-conjugated dopamine) should not have recognized non-glutaraldehyde fixed dopamine, but it did. Any fixative has the potential to alter experimental results, particularly in the case of extended fixation times that tend to mask antigenic sites, and this poses problems the investigator must be aware of and be ready to counter. However, it would seem that in cases where conjugated antigens are used to produce antibodies, extra care must be taken and results interpreted with the proper perspective and caution.

The association of dopamine with GH cells during one phase of development and with GTH cells during another phase of development, is particularly interesting. Other studies have linked this neurotransmitter to both of these cell types, as previously mentioned, but this is the first indication that physiologically, *in vivo*, dopamine displays a differential association with pituitary cell types that is dependent on the physiological state of the organism. This is a thought that should be kept in mind as further explorations take place. The localization of dopamine in the prolactin-producing cells of the platyfish pituitary gland is in keeping with reports that claim dopamine to be a prolactin release-inhibiting factor in both mammals and teleosts (see Grau and Helms, 1990).

Ir-SP, -BK, and -VIP, were also not localized in the *Xiphophorus* brain or pituitary gland. These negative results may be due to several factors, including the possibility that:

1. these substances do not exist in teleosts,
2. that they exist, but have structures that are very different from the mammalian homologs used to generate our antisera,
3. that they exist in some teleosts, but not in *Xiphophorus*,
4. that our methods were inadequate to detect their presence,
5. their presence may be physiologically restricted to developmental stages, ages or even to anatomical structures not examined in this study.

VIP, BK, VIP, GABA and GLU have received a lot of attention from investigators of mammalian systems but very little from investigators of non-

mammalian systems. Despite our negative findings, it is still our belief that newer investigative probes will demonstrate the importance of at least some of these factors in teleost reproductive system development and function.

It is, by now, well-documented and generally well-accepted that the allelic series at the *P* locus on the sex chromosome of *Xiphophorus maculatus* controls the age and size at which platyfish reach sexual maturity. The mechanism of *P* gene activity remains, for the moment unknown. However, strides in resolving this mystery are being taken. The isolation and cloning of this gene is underway through the joints efforts of at least two major laboratories (Schreibman, *et al.*, 1994). It is hoped that these efforts will elucidate the means by which this genetic locus exerts its control on the maturational process.

This report on variant forms of ir-GnRH and -GTH in the *Xiphophorus* brain, and our findings on the temporal appearance and distribution of neural peptides (Magliulo-Cepriano and Schreibman, 1993; Magliulo-Cepriano, *et al.*, 1993, 1994), supports and enhances our contention that there is a clearly defined sequence of developmental events, under genetic control, that orchestrate the spatial and temporal events of reproductive system development.

Previous data had suggested that the *P* gene activates a "switch" that initiates puberty (Halpern-Sebold and Schreibman, 1983). This switch might reside in one of the GnRH-containing nuclei of the brain. The NOR is a likely candidate since it is the first of the brain centers known to contain ir-GnRH. The results of this study indicate that, if *P* gene activity is a result of NOR-GnRH interactions, then it is most likely that

either, or both the mammalian and salmon form of the GnRH molecule are involved in initiating maturational events. This report has also established that the GnRH neurons of the NOR interact with ir-NPY and -FMRF-amide. The possibility exists that *P* gene activity resides with these neuroregulatory peptides and their functions within this nucleus.

The NPP is also a candidate, particularly if the switch is actually a release from inhibition rather than a stimulus. In this case, the activity of NPY in this nucleus deserves closer attention. Ir-NPY is present in the NPP 0.5 mos old animals but not in animals at the age of 1.5 mos, which marks the onset of puberty in the JP163 platyfish. The presence of NPY in this nuclei may act as a neuromodulator, decreasing the sensitivity of neurons and thus inhibiting the flow of neural signals to the pituitary gland.

The NLT is also a candidate, although not a popular one. However, the NLT, in this study, was found to contain ir-androgen receptors. If *P* gene activity results in neuromodulatory effects that alter the sensitivity of brain neurons, then the influence of gonadal steroids on NLT neurons involved in the orchestration of puberty could prove to be highly significant.

This study has shown that the NPO is yet another candidate for *P* gene activity. The changes in the size and number of ir-GnRH containing preoptic neurons has been shown to influence the timing of sexual maturation (Grober, *et al.*, 1994). One thought-provoking finding of this report is the demonstration that ir-GAL appears in

the NPO at the onset of puberty and that ir-FMRF-amide, -mGnRH and -sGnRH containing fibers from NOR neurons project to this nucleus.

Elucidating the nature and pathways of *P* gene activity has been slow and methodical. The findings of this study further demonstrate that sexual maturation is a complicated series of interactions involving a genetic component, at least three major organs, a number of neuropeptides, multiple forms of GnRH and GTH, in addition to a responsiveness to the internal and external environment. We have established a biological calendar for the various neuroendocrine humors and discussed their possible functions in discrete brain nuclei believed to be involved in the maturational process. However, there is still considerable ground to cover before we can fully comprehend the role of the genome, the environment, the brain and the endocrine system in the development and maturation of the reproductive system.

Appendix

Table 1: Primary structures of the eight known GnRH molecules.

Lamprey-III	pGlu - His - Trp - Ser - His - Asp - Trp - Lys - Pro - Gly - NH_2
Lamprey-I	pGlu - His - Tyr - Ser - Leu - Glu - Trp - Lys - Pro - Gly - NH_2
Dogfish	pGlu - His - Trp - Ser - His - Gly - Trp - Leu - Pro - Gly - NH_2
Catfish-I	pGlu - His - Trp - Ser - His - Gly - Leu - Asn - Pro - Gly - NH_2
Salmon	pGlu - His - Trp - Ser - Tyr - Gly - Trp - Leu - Pro - Gly - NH_2
Chicken-II	pGlu - His - Trp - Ser - His - Gly - Trp - Tyr - Pro - Gly - NH_2
Chicken-I	pGlu - His - Trp - Ser - Tyr - Gly - Leu - Gln - Pro - Gly - NH_2
Mammal	pGlu - His - Trp - Ser - Tyr - Gly - Leu - Arg - Pro - Gly - NH_2

Table 2: Dilutions and Sources of Antisera

Antiserum Against:	Source:	Dilution:
Porcine ACTH (1-39)	NIAMDD-NIH Batch#2	1:500
Synthetic AVT	K.Lederis, University of Calgary, Canada	1:400
Synthetic BK	Sera-lab, Los Angeles, CA. Lot# ASOJ4113	1:100
Dopamine (Dopamine-Glutaraldehede)	Chemicon, Temecula, CA. Lot# 12-89/AB122S	1:200
Dopamine (Dopamine-Formaldehyde)	M. Geffard, Universite Bordeaux, France.	1:400
Porcine DYN	Peninsula Laboratories, Belmont, CA. Lot# 019241-1	1:200
Synthetic FMRF-amide	Incstar, Stillwater, MN. Lot# 9049025	1:300
Porcine GAL	Chemicon, Temecula, CA. Cat# AB1985	1:500
GABA (GABA-Gluteraldehyde)	Chemicon, Temecula, CA. Cat# AB141	1:100
Glutamate (Glutamate-Gluteraldehyde)	Chemicon, Temecula, CA. Cat# AB133	1:100
Coho Salmon Beta GTH I	P. Swanson, University of Washington, Seattle, U.S.A.	1:1000
Coho Salmon Beta GTH II	P. Swanson, University of Washington, Seattle, U.S.A.	1:500
Croaker Beta GTH	P.Thomas, University of Texas, P. Aransas, U.S.A.	1:5000
Chicken II GnRH (chIIGnRH)	H.J.Th. Goos, University of Utrecht, The Netherlands	1:1000
Lamprey I GnRH (lGnRH)	S.Sower, University of New Hampshire, U.S.A.	1:5000

Mammalian GnRH	H.J.Th.Goos, University of Utrecht, The Netherlands	1:500
Salmon GnRH (sGnRH)	Y. Zohar, University of Maryland, Baltimore, U.S.A.	1:1000
Tilapia GH	S.Holtzman, Brookhaven National Laboratories, New York, U.S.A.	1:30000
Porcine NPY	Peninsula Labs. Belmont, CA. Lot# 015575-6	1:200
Synthetic NT	Incstar, Stillwater, MN. Lot# 8937050	1:500
Salmon PRL	P.Swanson, University of Washington, Seattle, Washington, U.S.A.	1:2000
Serotonin (Serotonin-BSA)	Immunonuclear, Stillwater, MN. Lot# 8310027	1:400
Cod SL	M.Rand-Weaver, University of West London, United Kingdom	1:1000
Mammalian SP	Peninsula Laboratories, Belmont, CA. Lot# 060955-1	1:100
Human TSH	Accurate Chemical & Scientific Corp. Westbury, N.Y. Lot# 123-p	1:1500
Mammalian VIP	Peninsula Laboratories, Belmont CA. Lot# 020944-1	1:100

Table 3: Results of the variant forms of GnRH/GTH absorption study. A minus mark indicates that the immunoreactivity was eliminated by the absorption. A plus mark indicates that the immunoreactivity was unaffected by the absorption. A plus and a minus together indicate that the immune reaction was decreased by the absorption; in these cases the absorbed antibody was used for the localization.

Antisera / Antigen	Anti-Salmon	Anti-Mammal	Anti-Chick II	Anti-Lampr I	Anti-GTH I	Anti-GTH II
salmon GnRH	-	+/-	+	+	+	+
mammal GnRH	+/-	-	+	+/-	+/-	+
chick II GnRH	+	+	-	+	+	+
lamprey I GnRH	+	+	+	-	+	+
GTH I	+	+	+	+	-	+
GTH II	+	+	+	+	+	-

Table 4. Distribution of immunoreactive neuropeptides in the brain and pituitary gland of *Xiphophorus maculatus*.

Peptide / Age Group	FMRF-amide B	FMRF-amide P	Galanin B	Galanin P	Neurotensin B	Neurotensin P	Dynorphin B	Dynorphin P	Neuropeptide Y B	Neuropeptide Y P
2 day old (neonatal)	NOR	NH	-	NH CPD	-	-	OL	RPD CPD PI	NOR VT T	NH RPD CPD PI
0.5 mos old (immature)	NOR NPO	NH RPD	-	NH CPD RPD PI	NLT	-	OL	RPD CPD PI	NOR VT NPP T	NH RPD CPD PI
1.5 mos old (onset of puberty)	NOR NLT NPO NPP	NH RPD	NPO NPP NLT	NH CPD RPD PI	NLT	CPD RPD PI	OL	RPD CPD PI	NOR VT T	NH RPD CPD PI
3.0 mos old (mature)	NOR NLT NPO NPP	NH RPD	NPO NPP NLT	NH CPD RPD PI	NLT	CPD RPD PI	OL	RPD CPD PI	NOR VT T	NH RPD CPD PI

Table 5: Localization of variant forms of immunoreactive GnRH and GTH in the brain and pituitary gland of platyfish.

Peptide / Age Group	GTH I		GTH II		sGnRH		mGnRH		chGnRHII		lGnRH	
	B	P	B	P	B	P	B	P	B	P	B	P
Two Day old (Neonate)	-	PI	-	-	-	PI	-	CPD PI	-	-	-	PI
0.5 mos old (Immature)	-	PI CPD	-	-	-	PI CPD	-	PI CPD	-	-	-	PI
1.5 mos old (Puberty)	-	PI CPD	-	CPD	NOR	PI CPD	NOR	PI CPD	-	-	-	PI CPD
3 & 10 mos old (Mature)	-	PI CPD	-	CPD PI	NOR NPP NLT	PI CPD	NOR NPP NLT	PI CPD	NLT NPP NPO	PI CPD	-	PI CPD

Figure 1: This diagram suggests the structural and functional relationship among the various components of the brain-pituitary-gonad axis in *Xiphophorus* and the select sites where a variety of cues may affect this axis. [Taken from Schreibman and Margolis-Nunno, 1989.]

Figure 1

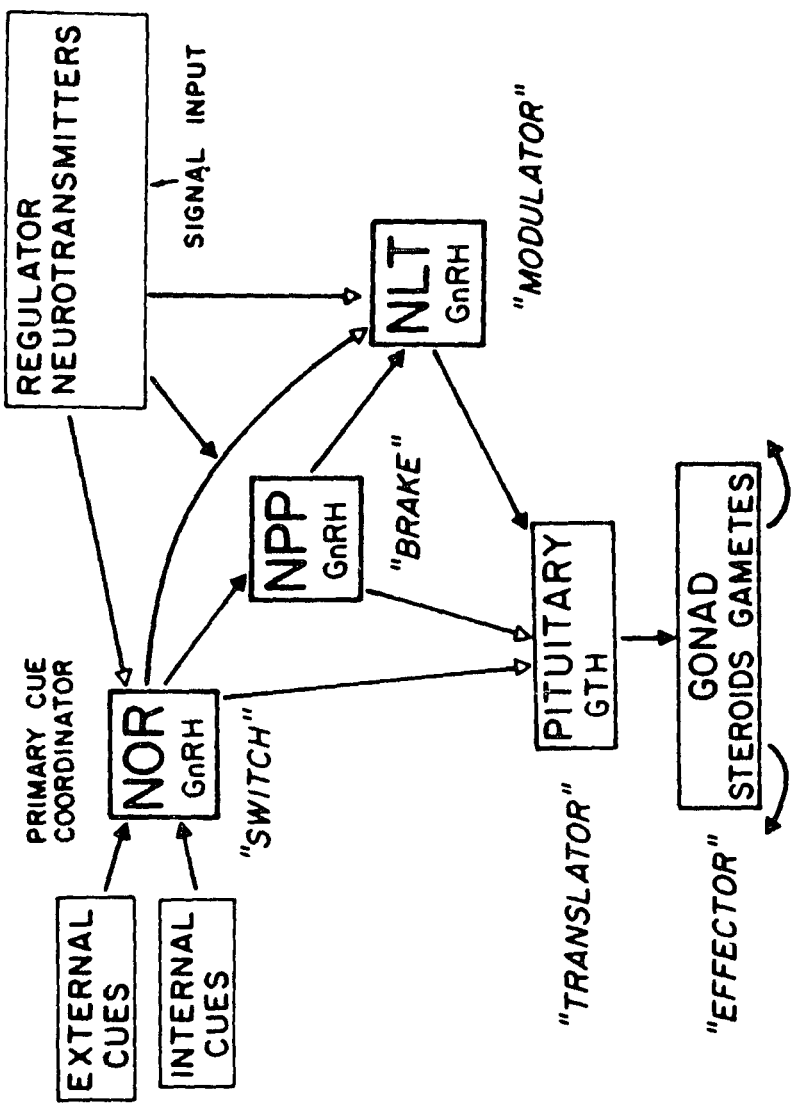
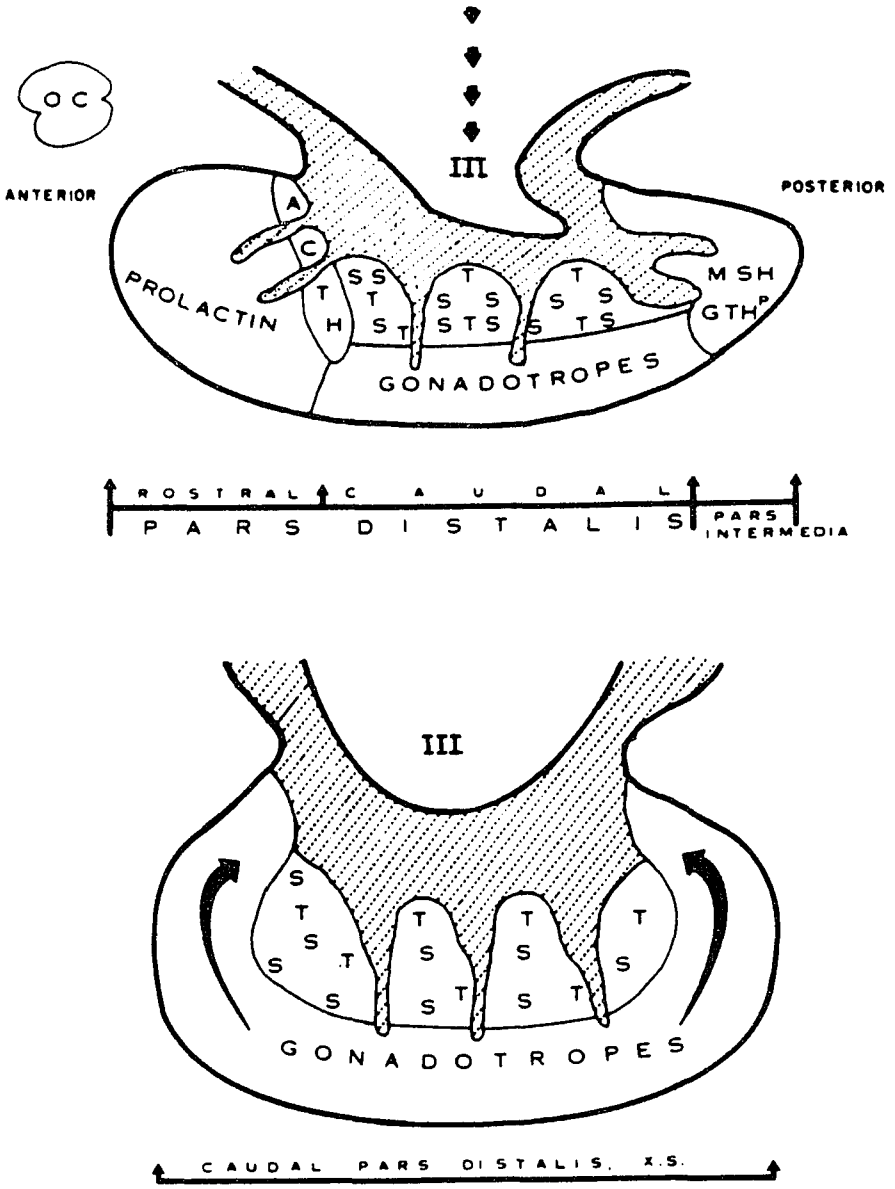

REGULATOR NEUROTRANSMITTERS
SIGNAL INPUT
NLT GnRH
"MODULATOR"
NPP GnRH
"BRAKE"
PRIMARY CUE COORDINATOR
NOR GnRH
"SWITCH"
PITUITARY GTH
"TRANSLATOR"
GONAD STEROIDS GAMETES
"EFFECTOR"
EXTERNAL CUES
INTERNAL CUES

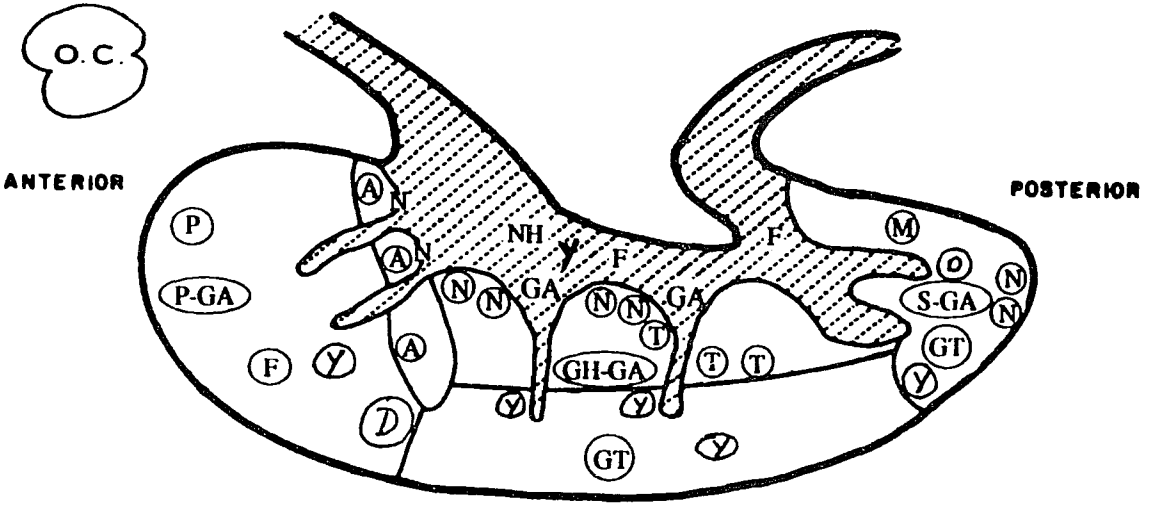
Figure 2: Schematic representation of a mid-sagittal (top) and a transverse (middle) section of the pituitary gland of a mature platyfish depicting the distribution of endocrine cells within the gland. The immature gland has all the components of the mature gland except for the zone of gonadotropin cells in the ventral CPD. Gonadotropin cells in the immature platyfish pituitary are found only in the extreme lateral of the CPD [Taken from Schreibman, 1986.]

Figure 2



KEY: ACTH - corticotropes (ACTH)
S - somatotropes (GH)
T - thyrotropes (TSH)
MSH - melanocyte stimulating hormone
GTH - gonadotropin hormone
III- third ventricle

Figure 3: Schematic representation of a mid-sagittal section of an adult platyfish pituitary gland illustrating the immunocytochemical distribution of ir-neuropeptides, and their colocalization with pituitary hormones, within the gland.



KEY: A - ACTH
D - dynorphin
F - FMRF-amide
G - growth hormone
GA - galanin
GT - gonadotropin
N - neurotensin
P - prolactin
S - somatolactin
T - thyrotropin
Y - neuropeptide Y

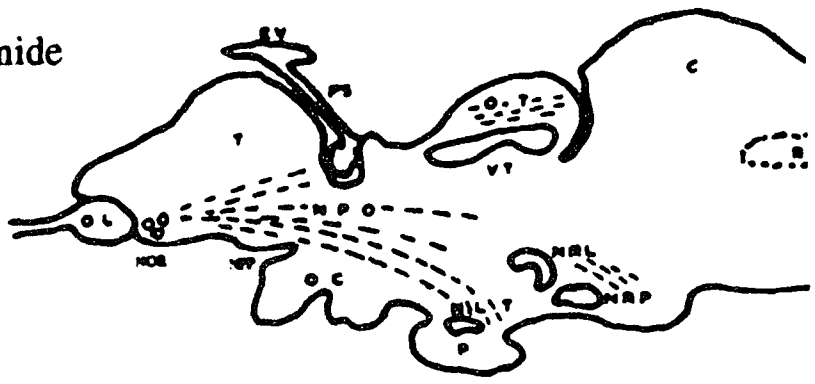
Figure 3

Figure 4: Drawing of a mid-sagittal section of the platyfish brain illustrating the immunocytochemical distribution of:

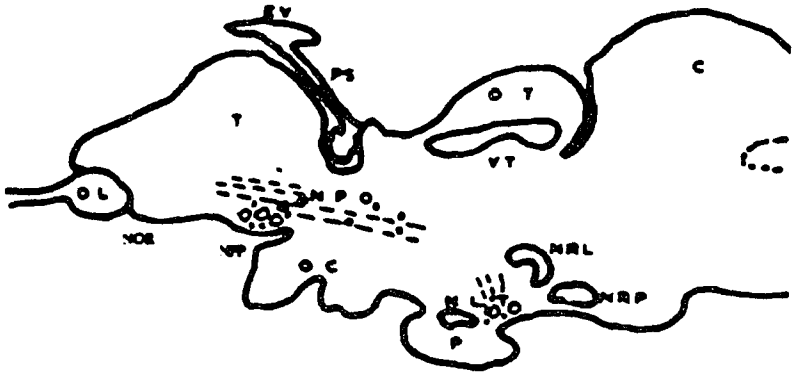
a) FMRF-amide b) GAL c) NT d) NPY

Figure 4

a) FMRF-amide



b) GAL



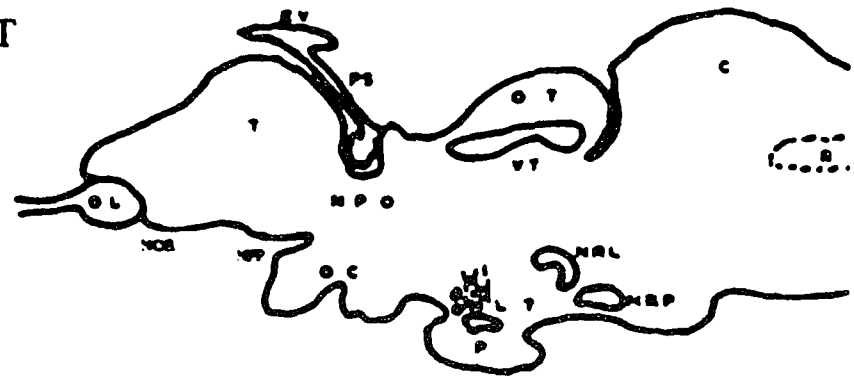
KEY: broken lines - tracts
open circles - perikarya
closed circles - axonal endings

Figure 4: Drawing of a mid-sagittal section of the platyfish brain illustrating the immunocytochemical distribution of:

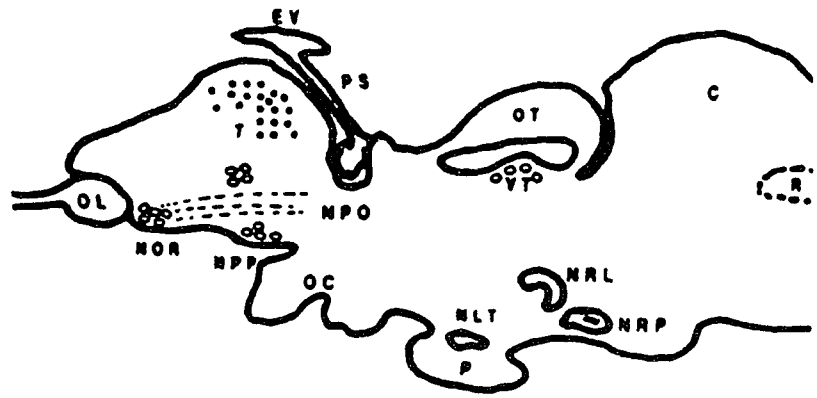
a) FMRF-amide b) GAL c) NT d) NPY

Figure 4

c) NT



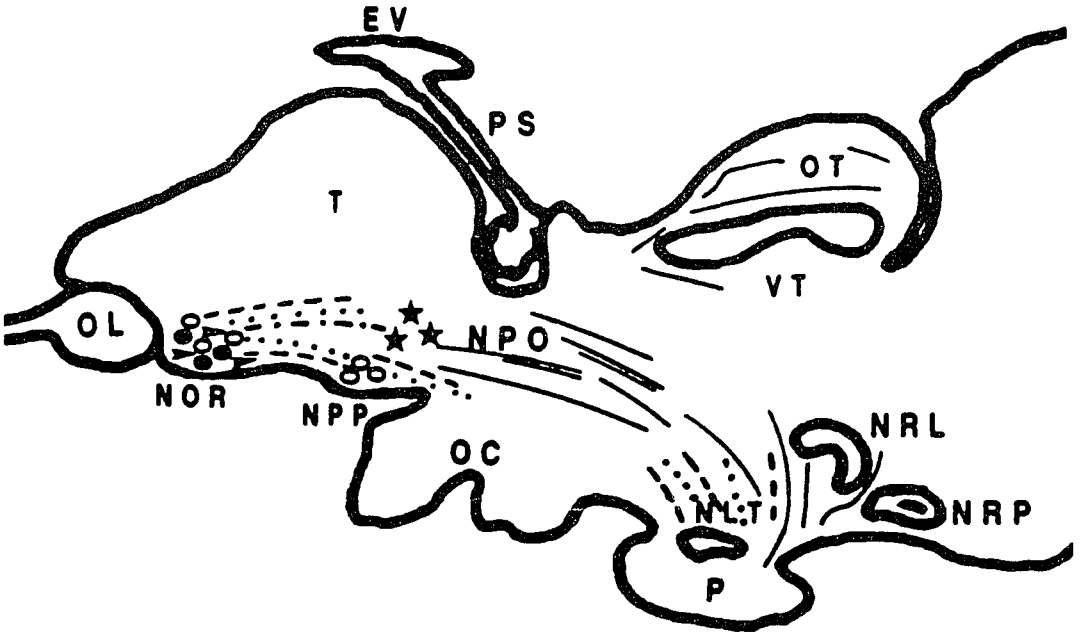
d) NPY



KEY: broken lines - tracts
open circles - perikarya
closed circles - axonal endings

Figure 5: Drawing of a mid-sagittal section of the platyfish brain illustrating the immunocytochemical distribution of variant forms of GnRH.

Figure 5



KEY: stars - chIIGnRH perikarya
empty ovals - sGnRh perikarya
arrowheads - mGnRH perikarya
filled ovals - colocalization of s and mGnRH
solid lines - chIIGnRH tracts
broken lines - sGnRH tracts
dotted lines - mGnRH tracts
alternating broken and dotted lines - s and mGnRH tracts

Figures 6 to 36, unless otherwise indicated, are mid-sagittal sections with anterior to the left.

Figure 6: Ir-FMRF-amide in perikarya and processes of NOR neurons. 520X

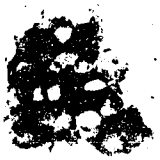
Figure 7: (Anterior is to the right.) Ir-FMRF-amide-containing fibers extending from NOR (star) neurons and traversing the NPO (N) along one pathway and extending towards the NPP in another (arrowheads). 70X

Figure 8: (Anterior is to the right.) Higher magnification of the NOR region of Figure 7. Note ir-perikarya in the NOR (arrowhead). 400X

Figure 9: Ir-GAL in all three regions of the pituitary gland. 350X

Figure 10: Distribution of ir-GAL in all regions of the adenohypophysis. Note intense immunoreaction in the CPD (arrowhead) where ir-GAL was found to colocalize with ir-GH. 300X

Figure 11: Ir-NT in axonal processes and terminal fields of the posterior NLT. 500X



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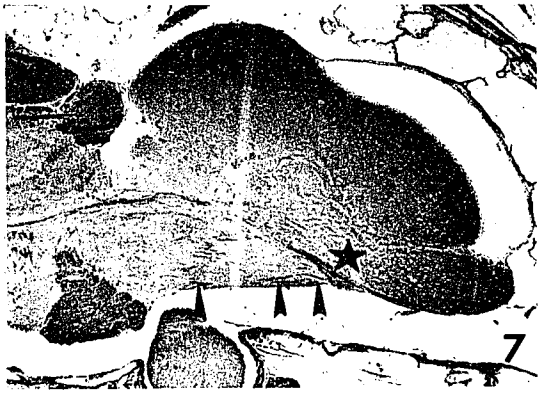

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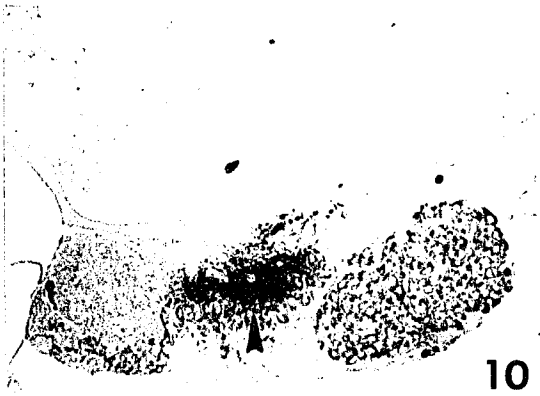

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Figure 12: Ir-NT within cells of the CPD (C) and in granules in the RPD (R). 320X

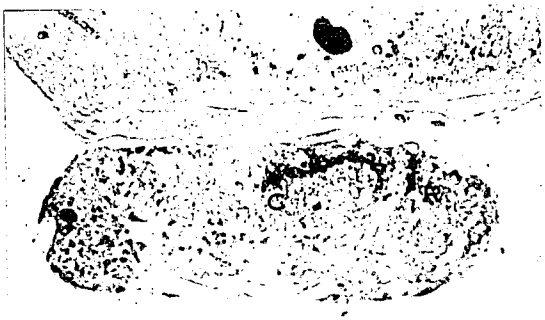
Figure 13: Intense ir-NT in the PI of a sexually mature fish. 350X

Figure 14: Transverse section. Bilateral clusters of ir-NPY neurons in the NOR (arrowheads). 200X

Figure 15: Ir-NPY granules in axonal processes and terminal fields in the medial telencephalon. 400X

Figure 16: Ir-NPY in the pituitary gland. 220X

Figure 17: Ir-DYN in the olfactory bulb. 200X



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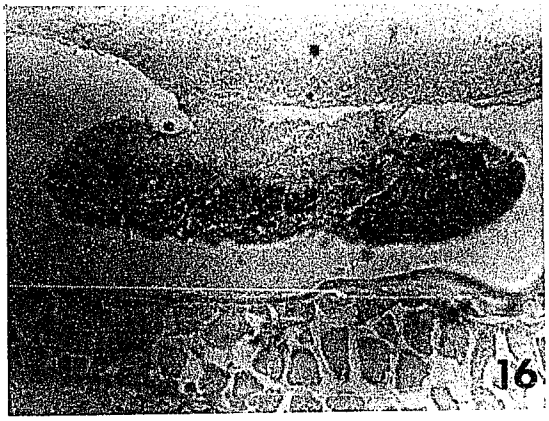

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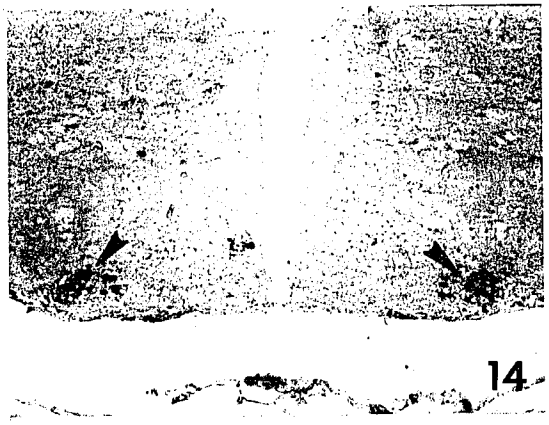

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Figure 18: Ir-DYN in cell clusters in the PI (P) and in the RPD (arrowhead). 310X

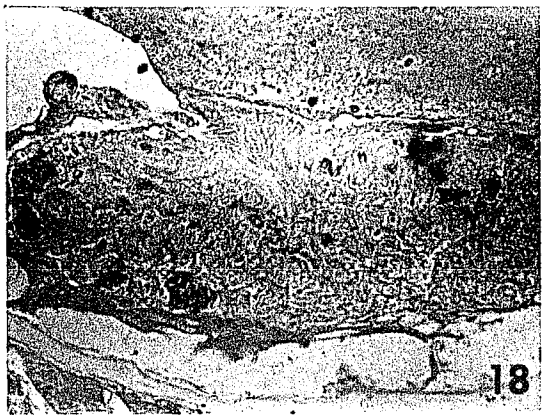
Figure 19: Ir-sGnRH in the NOR (arrowhead), the NPP (P) and in tracts between the two nuclei. 200X

Figure 20: Immunoreactive tracts spanning the NPO. Ir-sGnRH and -mGnRH were found to colocalize in some of the fibers in this pathway. 200X

Figure 21: Ir-sGnRH in the NPP. Note that the immunoreactivity appears both within the cytoplasm of neurons and as distinct ir-granules on cell and axonal membranes (arrowheads). 350X

Figure 22: Intense ir-sGnRH in the medial PI of a 0.5 mos old platyfish. 300X

Figure 23: Ir-sGnRH in the CPD of a mature platyfish. 310X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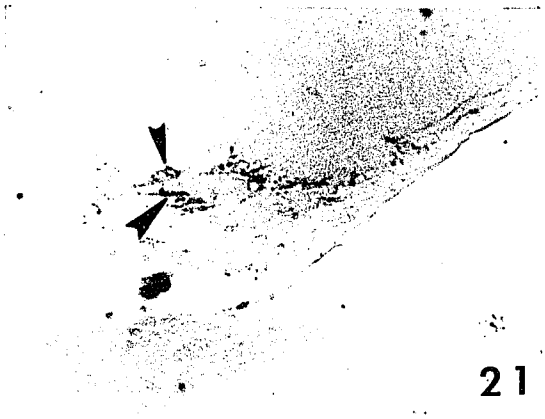
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Figure 24: Ir-mGnRH in a lateral section of the pituitary gland of a 0.5 mos old platyfish. CPD (C), PI (P). 310X

Figure 25: Ir-mGnRH in the CPD (C) and PI (P) of a mature platyfish pituitary. 260X

Figure 26: Ir-chIIGnRH in a cluster of fibers in the NPO of a mature platyfish brain. 600X

Figure 27: Ir-chIIGnRH in the CPD of a mature platyfish pituitary gland. 280X

Figure 28: Ir-lGnRH in the CPD (C) and PI (P) of a 0.5 mos old platyfish. 320X

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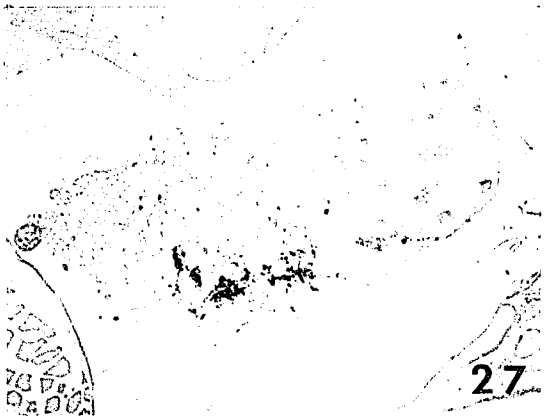

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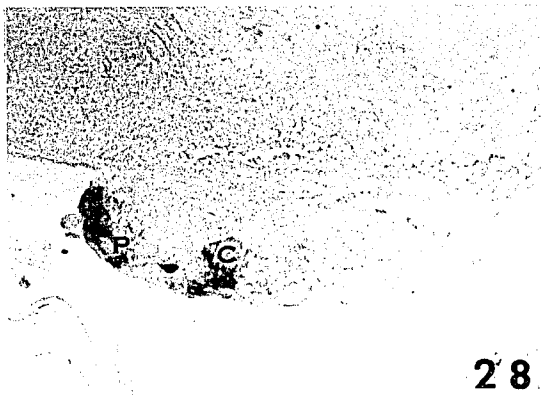

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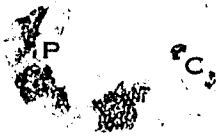

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Figure 29: Ir-lGnRH in the CPD (C) and PI (P) of a mature platyfish. 280X

Figure 30: Ir-GTH I in the PI (P) and CPD (C) of a 1.5 mos old platyfish. Note that while there appears to be a greater number of immunoreactive cells in the PI, the reaction in the CPD is of greater intensity. 190X

Figure 31: Ir-GTH I in a mature platyfish pituitary. At this age an abundance of immunoreactive material is seen in the ventral CPD (C). 220X

Figure 32: Ir-GTH II in the CPD (C) of a 1.5 mos old platyfish. Note the absence of immunoreactivity in the PI (P). Compare to the distribution of ir-GTH I in Fig. 30, an adjacent section of the same animal. 200X

Figure 33: Ir-GTH II in the CPD of a mature platyfish. Compare with figure 32. 210X

Figure 34: Ir-androgen receptors in the NLT of a mature platyfish. 400X

Figure 35: Higher magnification of the NLT region of a mature platyfish. Note the immunoreactivity in nuclei of NLT neurons. 600X

Figure 36: Ir-androgen receptors in the PI (P) and CPD (C) of a 1.5 mos old platyfish. Note the immunoreactivity occurring in both the cytoplasm and in nuclei (arrowheads). 350X

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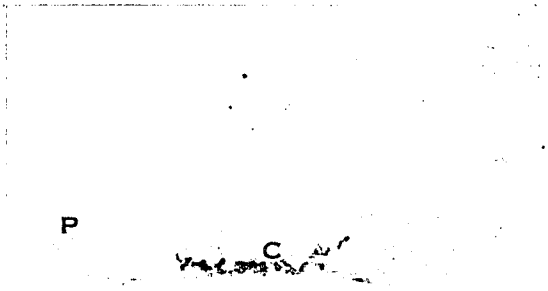

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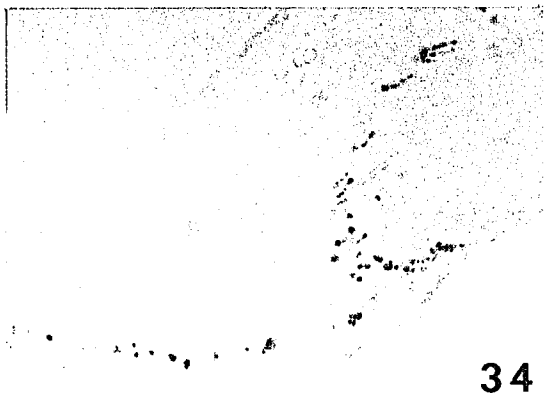

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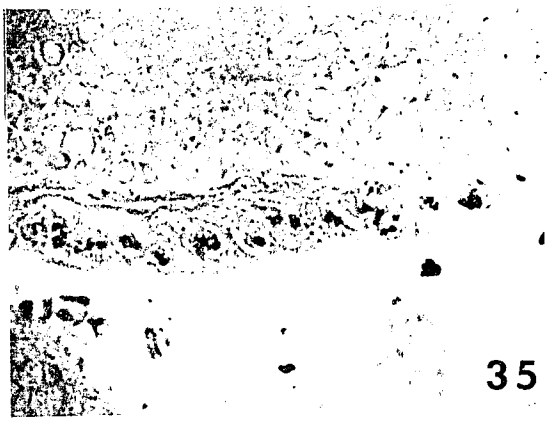

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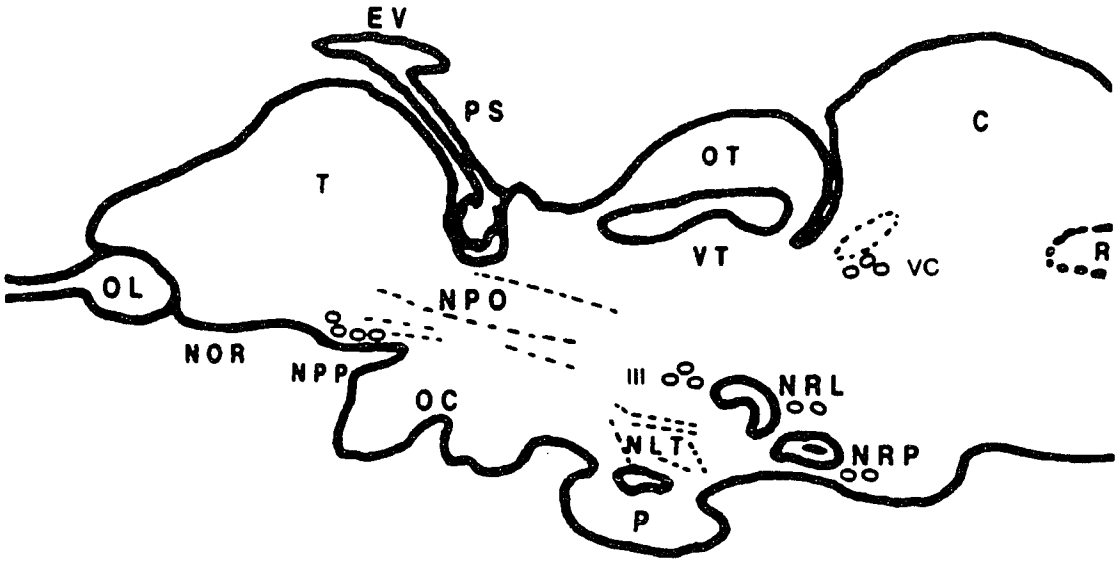
Figure 37: Schematic representation of the distribution of ir-serotonin in the brain of a mature platyfish.

Figure 37



KEY: broken lines - tracts
open circles - perikarya

Figure 38: The effect of administration of exogenous NPY on the percent increase in the standard length of immature (Group I) platyfish.

Figure 38

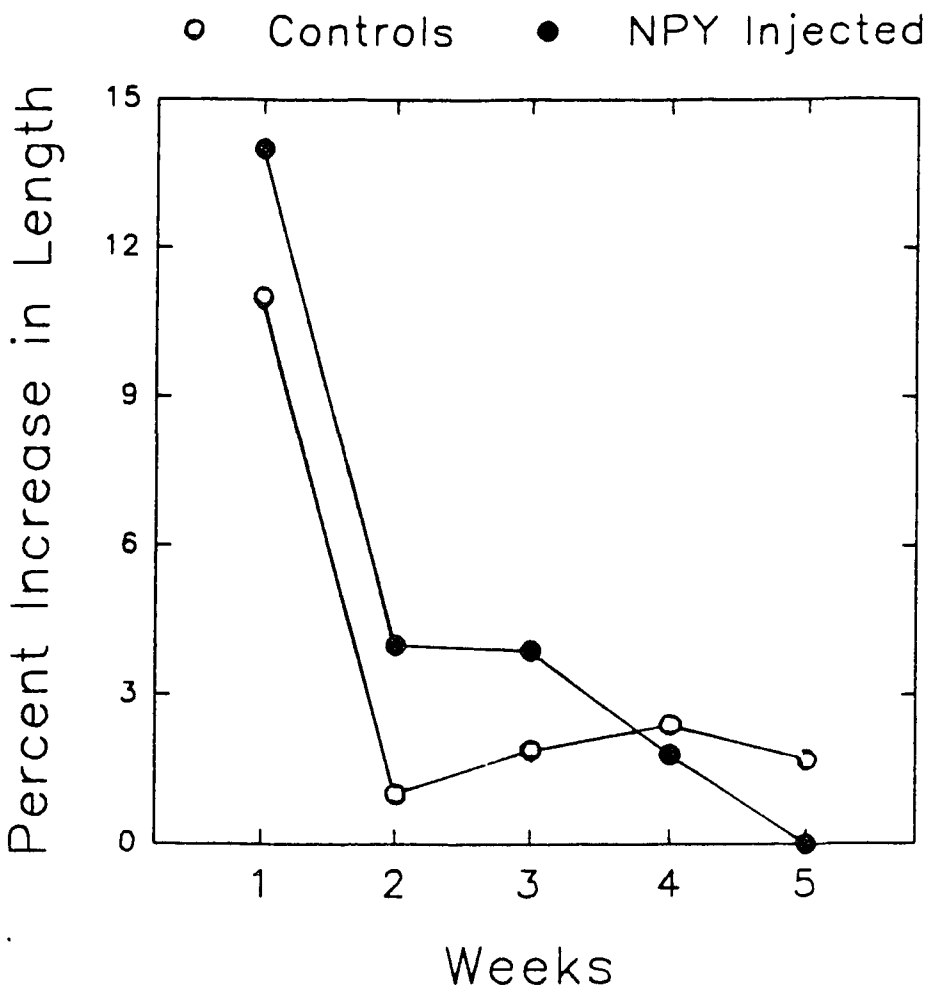
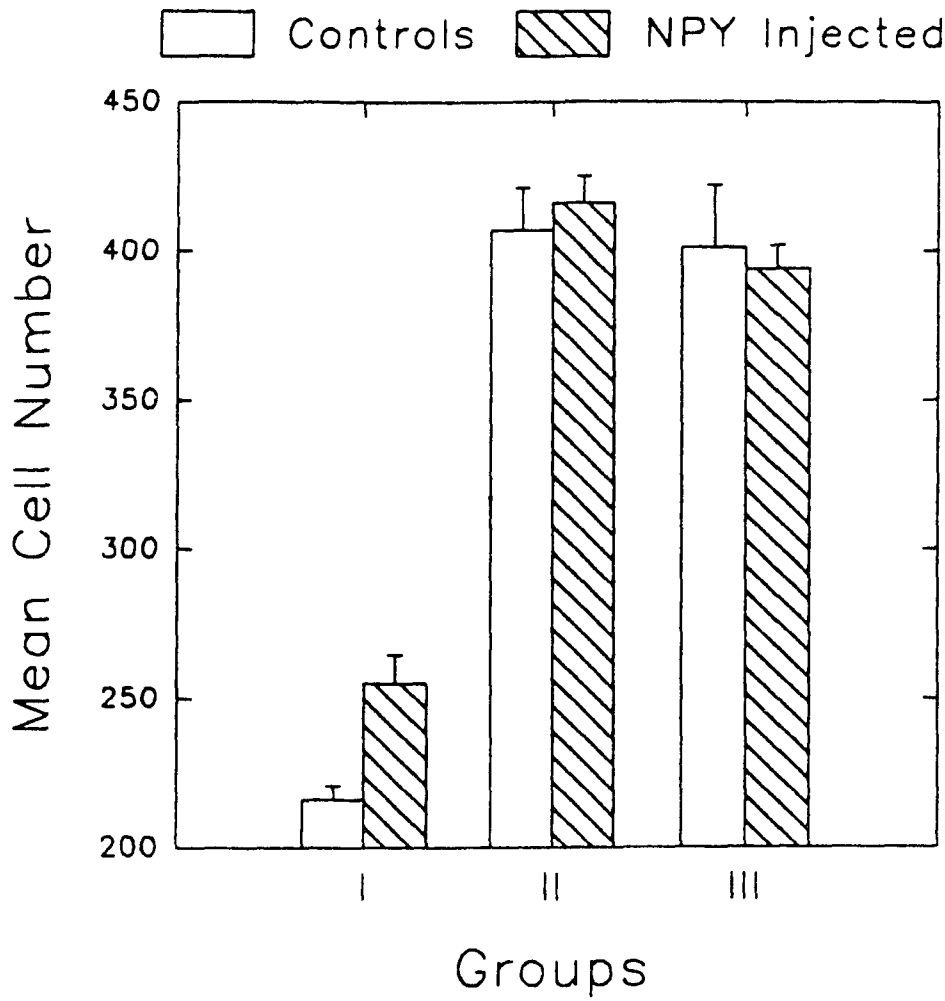

Controls
NPY Injected
Percent Increase in Length
15
12
9
6
3
0
1
2
3
4
5
Weeks

Figure 39: The effect of administration of exogenous NPY on the number of ir-growth hormone containing cells in the platyfish pituitary gland.

Figure 39



KEY: Group I - immature animals
Group II - mature females
Group III - mature males

REFERENCES

Adelman, J.P., Mason, A.J., Hayflick, J.S., Seeburg, P.H. (1986) Isolation of the gene and hypothalmic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin releasing-inhibiting factor in human and rat. *Proc. Natl. Acad. Sci. U.S.A.* 83:179-183.

Ahmed,C.E., Dees, W.L., Ojeda, S.R. (1986) The immature rat ovary is innervated be vasoactive intestinal peptide (VIP)-containing fibers and responds to VIP with steroid secretion. *Endocrinol.* 118:1682-1689.

Amano, M., Oka, K., Aida, K., Okumoto, N., Kawashima, S., Hasegawa,Y. (1991) Immunocytochemical demonstration of salmon GnRH and chicken GnRH-II in the brain of masu salmon (*Oncorhynchus masou.) J. Comp. Neurol.* 314:587-597.

Aronin, N., Coslovsky, R., Leeman, S.E. (1986) Substance P and neurotensin: their roles in the regulation of anterior pituitary function. *Annu. Rev. Physiol.* 48:537.

Bailey, P. and Bremer, F. (1921) Experimental diabetes insipidus. *Arch. Intern. Med.* 28:773.

Ball, G.F., Faris, P.L., Wingfield, J.C. (1986) Immunohisto-chemical localization of neuropeptides in two species of wild songbird. I. Hypothalamic distribution. *Proc. Soc. Neurosci.* (abstract) 12:290.

Barnett, F.H., Sohn, J., Reichlin, S., Jackson, I.M.D. (1982) Three luteinizing hormone-releasing hormone like substances in a teleost fish: none identical with the mammalian LH-RH decapeptide. *Biochem. Biophys. Res. Commun.* 1051:209-216.

Batten, T.F.C., Cambre, M.L., Moons, L., Vandesande, F. (1990). Comparative distribution of neuropeptide-immunoreactive systems in the brain of the green molly, *Poecilia latipinna. J. Comp. Neurol.* 302:893-919.

Batten, T. F. C., Moons, L., Cambre, M., and Vandesande, F. (1990a). Anatomical distribution of galanin-like immunoreactivity in the brain and pituitary of teleost fishes. *Neurosci. Lett.* 111:12-17.

Batten, T. F. C., Marivoet, S., and Vandesande, F. (1987). Neurotensin-like immunoreactivity in the pituitary and hypotalamus of bony fishes. *Peptides* 8:135-143.

Bennis, M., Dubourg, P., Gamrani, H., Calas, A., Kah, O. (1989) Existence of a GnRH immunoreactive nucleus in the doral midbrain tegmentum of the chameleon. *Gen. Comp. Endocrinol.* 75:195-203.

Besson, J., Rotsztejn, W., Laburthe, M., Epelbaum, J., Beaudet, A., Kordon, C., Rosselin, G. (1979) Vasoactive intestinal peptide (VIP): brain distribution, subcellular localization and effect of deaferentiation of the hypothalamus in rats. *Brain Res.* 165:79-85.

Bond, C.T., Francis, R.C., Fernald, R.D., Adelman, J.P. (1991) Characterization of complimentary DNA encoding the percursor for gonadotropin-releasing hormone and its associated peptide from a teleost fish. *Mol. Endocrinol.* 5:931-937.

Borg B., Goos, H.J.Th., Terlou, M. (1982) LHRH-immunoreactive cells in the brain of the three spined stickleback, *Gasterosteus aculeatus* L. (*Gasterosteidae*). *Cell Tissue Res.* 226:695-699.

Breton, B., Mikolajczyk, T., Popek, W., Bieniarz, K., Epler, P. (1991) Neuropeptide Y stimulates *in vivo* gonadotropin secretion in teleost fish. *Gen. Comp. Endocrinol.* 84:277-283.

Brown, E.R., Harlan, R.E., Krause, J.E. (1990) Gonadal steroid regulation of substance P (SP) and SP-encoding messenger ribonucleic acids in the rat anterior pituitary and hypothalamus. *Endocrinol.* 126:330.

Brownstein, M. J., and Mezey, E. (1986). Multiple chemical messengers in hypothalamic magnocellular neurons. *In* "Coexistence of neuronal messengers: A new principle in chemical transmission" Vol. 68, (T. Hokfelt, K. Fuxe, and B. Pernow, eds), pp. 161-168, Elsevier, New York.

Carraway, R. E., and Leeman, S. E. (1975). The amino acid sequence of a hypothalamic peptide, neurotensin. *J. Biol. Chem.* 250:1912-1918.

Carraway, R. E., and Leeman, S. E. (1973). The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. *J. Biol. Chem.* 248:6854-6861.

Carraway, R. E., Ruane, S. E., and Kim, H. K. (1982). Distribution and immuncytochemical character of neurotensin-like material in representative vertebrates and invertebrates: apparent conservation of the COOH-terminal during evolution. *Peptides* 1:115-123.

Chabot, J.G., Enjalbert, A., Pelletier, G., Dubois, P.M., Morel, G. (1988) Evidence for a direct action of neuropeptide Y in the rat pituitary gland. *Neuroendocrinol.* 47:511-517.

Chang, J.P., Jobin, R.M., Wong, A.O.L. (1993) Intracellular mechanisms mediating gonadotropin and growth hormone release in the goldfish, *Carassius auratus. Fish Physiol. Biochem.* 11:25-33.

Chiba, A., Oka, S., Honma, Y. (1991). Immunocytochemical distribution of FMRF-amide-like substances in the brain of the cloudy dogfish, *Scyliorhinus torazame. Cell. and. Tissue. Research.* 265:243-250.

Chihara, K., Arimura, A., Coy, D.H., Schally, A.V. (1978) Studies on the interaction endorphins, substance P, and endogenous somatostatin in growth hormone and prolactin release in rats. *Endocrinol.* 102:281.

Coe, I.R., von Schalburg, K., Adelman, J.P., Sherwood, N.M. (1992) Evolution of the gonadotropin-releasing hormone family: Peptides and genes. Program of the 74th annual meeting of The Endocrine Society, San Antonio, Tx. p. 97 *(abstract).*

Conlon, J.M., Hicks, J.H., Smith, D.D. (1990) Isolation and biological activity of a novel kinin ([Thr^6] bradykinin) from the turtle, *Pseudemys Scripta. Endocrinol.* 126:985-991.

Cornbrooks, E. B., and Parsons, R. L. (1991). Source of sexually dimorphic galanin-like immunoreactive projections in the teleost fish *Poecilia latipinna. J. Comp. Neurol.* 304:658-665.

Costa, M., Furness, J.B., Buffa, R., Said, S.I. (1980) Distribution of enteric nerve cells bodies and axons showing immunoreactivity for vasoactive intestinal peptide in the guinea pig intestine. *Neuroscience* 5:587-596.

Crawley, J. N., and Wenk, G. L. (1989). Co-existence of galanin and acetylcholine: Is galanin involved in memory processes and dementia? *Trends Neurosci.* 12:278-282.

Crosby, E. C., and Showers, M. J. C. (1969). Comparative anatomy of the preoptic and hypothalamic areas. *In* "The Hypothalamus", (W. Haymaker, E. Anderson, and W. H. J. Nauta, eds), pp. 61-135, Thomas, Springfield, Ill.

Crowley, W.R. and Kalra, S.P. (1988) Regulation of luteinizing hormone secretion by neuropeptide Y in rats: hypothalamic and pituitary actions. *Synapse* 2:276-281.

Crowley, W.R., Hassid, A., Kalra, S.P. (1987) Neuropeptide Y enhances the release of luteinizing hormone (LH) induced by LH-releasing hormone. *Endocrinology* 120:941-945.

Danger, J.M., Breton, B., Vallarino, M., Fournier, A., Pelletier, G., Vaudry, H. (1991) Neuropeptide Y in the trout brain and pituitary: Localization, characterization, and action on gonadotropin release. *Endocrinol.* 128:2360-2368.

Davis, R.E. and Kassel, J. (1983) Behavioral functions of the teleostean telencephalon. *In:* (Davis, R.E. and Northcutt, R.G., Eds.) Fish Neurobiology, Vol. 1. University of Michigan Press, Ann Arbor, pp. 237-263.

Davis, R.E., Morrell, J.I., Pfaff, D.W. (1977) Autoradiographic localization of sex steroid-concentrating cells in the brain of the teleost *Macropodus opercularis* (Osteichthyes: Belontiidae). *Gen. Comp. Endocrinol.* 33:496-505.

Davis, T. M. E., Burrin, J. M., Bloom, S. R. (1987). Growth hormone (GH) release in response to GH-releasing hormone in man is 3-fold enhanced by galanin. *J. Clin. Endocrinol. Metab.* 1248-1252.

Decavel, C. and van den Pol, A.N. (1990) GABA: A dominant neurotransmitter in the hypothalmus. *J. Comp. Neurol.* 302:1019-1037.

Delitala, G. (1991) Opioid peptides and pituitary function, basic and clinical aspects. *In*: (Motta, M., ed.) Brain Endocrinology, Second Edn. Raven Press, Ltd., New York.

Demski, L.S. (1983) Behavioral effects of of electrical stimulation of the brain. *In:* (Davis, R.E., and Northcutt, R.G., eds.) Fish Neurobiology, Vol. 2. University of Michigan Press, Ann Arbor. pp. 317-359.

Demski, L.S. (1984) The evolution of neuroanatomical substrates of reproductive behavior: sex steroid and LHRH-specific pathways including the terminal nerve. *Amer. Zool.* 24:809-830.

Demski, L.S., and Northcutt, R.G. (1983). The terminal nerve: a new chemosensory system in vertebrates? *Science* 220:435-437.

Demski, L.S., Bauer, D.J., Gerald, J.W. (1975) Sperm release evoked by electrical stimulation of the fish brain: A functional anatomical study. *J. Exp. Zool.* 191:215-232.

Dulka, J. G., Stacey, N. E., Sorensen, P. W., Van Der Kraak, G. J., Marchant, T. A. (1987). A sex pheromone system in goldfish: Is the nervus terminalis involved? *Ann. N.Y. Acad. Sci.* 411-420.

Dunn, I.C., Sharp, P.J., Sang, H.M. (1992) Evolution of GnRH peptides *(abs).* *In:* Proceedings of the British Neuroendocrine Group Meeting, Edinburgh, Scotland.

Ekstrom, P., Honkanen, T., and Ebbesson, S. O. E. (1988). FMRFamide-like immunoreactive neurons of the nervus terminalis of teleosts innervate both retina and pineal organ. *Brain. Res.* 460:68-75.

Enjalbert, A., Arancibia, S., Priam, M., Bluet-Pajot, M. T., Kordon, C. (1982). Neurotensin stimulation of prolactin secretion in vitro. *Neuroendocrinol.* 34:95-98.

Fuxe, K., Hokfelt, T., Said, S.I., Mutt, V. (1977) Vasoactive intestinal polypeptide and the nervous system: immunohistochemical evidence for localization in the central and peripheral neurons, particularly intracortical neurons in the cerebral cortex. *Neurosci. Lett.* 5:241-246.

Gaymann, W., and Martin, R. (1989). Immunoreactive galanin-like material in magnocellular hypothalamo-neurohypophysial neurones of the rat. *Cell Tissue Res.* 255:139-147.

Goos, H.J.Th. and Muranthanoglu, O. (1977) Localization of gonadotropin releasing hormone (GRH) in the forebrain of the trout *(Salmo gairdneri). Cell Tiss. Res.* 181:163-68.

Grau, E.G. and Helms, L.M.H. (1990) The tilapia prolactin cell - twenty-five years of investigation. *In:* (Epple, A., Scanes, C.G., Stetson, M.H., Eds.) Progress in Comparative Endocrinology, Wiley-Liss, pp. 534-540.

Gray, T.S. and Morley, J.E. (1986) Neuropeptide Y: anatomical distribution and possible function in mammalian nervous system. *Life. Sci.* 38:389-401.

Grober, M.S. and Bass, A.H. (1991) Neuronal correlates of sex/role change in labrid fishes: LHRH-like immunoreactivity. *Brain Behav. Evol.* 38:302-312.

Grober, M.S., Fox, S.H., Laughlin, C., Bass, A.H. (1994) GnRH cell size and number in a teleost fish with two male morphs: sexual maturation, final sexual status and body size allometry. *Brain Behav. Evol.* 43:61-78.

Grober, M.S., Bass, A.H., Meyers, D. (1992) GnRH cDNA sequence from the sexually polymorphic teleost fish, *Porichthys notatus. Proc. Soc. Neurosci.* (abstract) p.894.

Grobstein, D. (1948) Endocrine and developmental studies of gonopod diferentiation in certain poeciliid fishes. 1. The structure and development of the gonopod in *Platypoecilus maculatus. Univ. of Calif. Publ. Zool.* 47:1-22.

Halpern-Sebold, L., and Schreibman, M. P. (1983). Ontogeny of luteinizing hormone-releasing hormone containing centers in the brain of the platyfish (*Xiphophorus maculatus*) as determined by immunocytochemistry. *Cell Tissue Res.* 229:75-84.

Halpern-Sebold, L., Schreibman, M.P., Margolis-Nunno, H. (1986) Differences between early and late maturing genotypes of the platyfish (*Xiphophorus maculatus*) in the morphometry of their immunoreactive LHRH containing cells: A developmental study. *J. Exp. Zool.* 240:245-257.

Harris, G.W. (1961) The pituitary stalk and ovulation. *In:* (Vilee, C.A., Ed.) Control of Ovulation. Pergamon press, Elmsford, New York.

Holmqvist, B. I., and Ekstrom, P. (1991). Galanin-like immunoreactivity in the brain of teleosts: Distribution and relation to Substance P, vasotocin, and isotocin in the Atlantic salmon (*Salmo salar*). *J. Comp. Neurol.* 306:361-381.

Hulting, Anna-L., Meister, B., Carlsson, L., Hilding, A., Isaksson, O. (1991). On the role of the peptide galanin in the regulation of growth hormone secretion. *Acta. Endocrinol.* 125:518-525.

Hyde, J. F., Engle, M. G., Maley, B. E. (1991). Colocalization of galanin and prolactin within secretory granules of anterior pituitary cells in estrogen-treated Fischer 344 rats. *Endocrinology.* 129:270-275.

Idler, D.R. and Everard, B.A. (1987) Mammalian, salmon and chicken-like LHRH's from the hypothalami of winter flounder (*Pseudopleuronectes americanus*) as evidenced by chromatographic mobility and immunoreactivity. *In*: Reproductive Physiology of Fish. D.R. Idler, L.W. Crim, J.M. Walsh (eds.) Proceedings of the third international symposium on the reproductive physiology of fish. St. John's, New Foundland, Canada, p. 30.

Jacobs, B.L., Wilkinson, L.O., Fornal, C.A. (1990) The role of brain serotonin. *Neuropharmacol.* 3:473-479.

Jones, S.W. (1987) Chicken II luteinizing hormone-releasing hormone inhibits the M-current of bullfrog sympathetic neurons. *Neurosci. Lett.* 80:180-184.

Kah, O., Trudeau, V.L., Sloley, B.D., Chang, J.P., Dubourg, P., Yu, K.L., Peter, R.E. (1992) Influence of GABA on gonadotropin release in the goldfish. *Neuroendocrinol.* 55:396-404.

Kah, O., Trudeau, V.L., Yu, K.L., Chang, J.P., Peter, R.E. (1990) GABA and the neuroendocrine regulation of gonadotropin release in the goldfish. *Neuroendocrinol.* 52 (suppl. 1):93.

Kah, O., Dubourg, P., Martinoli, M.G., Rabhi, M., Gonnet, F., Geffard, M., Calas, A. (1987) Central GABAergic innervation of the pituitary in goldfish. A radioautographic and immunocytochemical study at the electron microscope level. *Gen. Comp. Endocrinol.* 67: 324-332.

Kah, O., Breton, B., Dulka, J.G., Nunez-Rodriquez, J., Peter, R.E., Corrigan, A., Rivier, J.E., Vale, W.W. (1986) A reinvestigation of the Gn-RH (gonadotropin-releasing hormone) systems in the goldfish brain using antibodies to salmon Gn-RH. *Cell Tissue Res.* 244:327-337.

Kah, O., Peter, R.E., Dubourg, P., Cook, H. (1983) Effects of monosodium L-glutamate on pituitary innervation in poldfish, *Carassius aurata. Gen. Comp. Endocrinol.* 51:338-346.

Kah, O., Chambolle, P., Dubourg, P., Dubois, M.P. (1982) Distribution of immunoreactive LHRH in the brain of the goldfish. *In:* (Richter, C.J.J. and Goos, H.J.TH., Eds.) Proceeding of the international symposium on reproductive physiology

of fish. Wageningen, the Netherlands. Center for Agricultural Publishing and Documentation, p. 56.

Kallman, K.D. (1989) Genetic control of size at maturity in *Xiphophorus. In:* (Meffee, G.K. and Snelson, F.F., Eds.) Ecology and Evolution in Livebearing Fishes *(Poeciliidae).* Prentice Hall, Englewood Cliffs, New Jersey, pp. 163-184.

Kallman, K.D. and Schreibman, M.P. (1973) A sex-linked gene controlling gonadotrope differentiation and its significance in in determining the age of sexual maturation and size of the platyfish, *Xiphophorus maculatus. Gen. Comp. Endocrinol.* 21:287-304.

Kalra, S.P. and Kalra, P.S. (1991) Steroid-peptide interactions in the endocrine brain: reproduction. *In*: (Motta, M. ed.) Brain Endocrinology, Second Edn. Raven Press, Ltd., New York.

Kato, Y., Chihara, K., Ohgo, S., Iwasaki, Y., Abe, H., Imura, H., (1976) Growth hormone and prolactin release by substance P in rats. *Life Sci.* 19:441.

Kaul, S. and Vollrath, L., (1974) The goldfish pituitary. 1. Cytology. *Cell Tiss. Res.* 154:211-230.

Kawauchi, H., Suzuki, K., Itoh, H., Swanson, P., Naito, N., Nagahama, Y., Nozaki, M., Nakai, Y., Itoh, S. (1989) The duality of teleost gonadotropins. *Fish Physiol. Biochem.* 7:29-38.

Kawauchi, H., Suzuki, K., Itoh, H., Swanson, P., Nagahama, Y. (1987) Duality of salmon pituitary gonadotropins. *In*: (Ohnishi, E., Nagahama, Y., Ishizaki, H. Eds.) Proceedings of the First Congress of the Asia and Oceania Society of Comparative Endocrinology. Nagoya, pp. 15-18.

Kaynard, A.H., Pau, K-Y.F., Hess, D.L., Spies, H.G. (1990) Third-ventricular infusion of neuropeptide Y suppresses luteinizing hormone secretion in ovariectomized Rhesus Macaques. *Endocrinology* 127:2347-2444.

Khan, I.A. and Thomas, P. (1993) Immunocytochemical localization of serotonin and gonadotropin-releasing hormone in the brain and pituitary gland of the atlantic croaker *Micropogonias undulatus. Gen. Comp. Endocrinol.* 91:167-180.

Khan, I.A. and Thomas, P. (1992) Stimulatory effects of serotonin on maturational gonadotropin release in the atlantic croaker, *Micropogonias undulatus. Gen. Comp. Endocrinol.* 88:388- 396.

Kim, Y.S., Stumpf, W.E., Sar, M. (1979) Topographical distribution of of estrogen target cells in the forebrain of Platyfish, Xiphophorus maculatus, studies by autoradiography. *Brain Res.* 170:43-59.

King, J.A. and Millar, R.P. (1993) Evolution of gonadotropin-releasing hormones. *Trends Endocrinol. Metab.* 3:339-346.

King, J.A. and Millar, R.P. (1986) Identification of His^5, Trp^7, Tyr^8-GnRH (chicken GnRH II) in amphibian brain. *Peptides* 7:827-834.

King, J.A. and Millar, R.P. (1985) Multiple molecular forms of gonadotropin-releasing hormone in teleost fish brain. *Peptides.* 6:689-694.

King, J.A. and Millar, R.P. (1982) Structure of chicken hypothalamic luteinizing hormone-releasing hormone I. Structural determination on partially purified material. *J. Biol. Chem.* 257:10722-10728.

King, J.A. and Millar, R.P. (1982a) Structure of chicken hypothalmic luteinizing hormone-releasing hormone II. Isolation and characterization. *J. Biol. Chem.* 257:10729-10732.

Klungland, H., Lorens, J.B., Andersen, O., Kisen, G.O., Alestrom, P. (1992) The Atlantic salmon prepro-gonadotropin-releasing hormone gene and mRNA. *Mol. Cell. Endocrinol.* 84:167-174.

Kuan, S.I., Judd, A.M., Jarvis, W.D., Login, I.S., MacLeod, R.M. (1990) Physiological and biochemical effects of bradykinin and *lys*-bradykinin in pituitary cells. *Molec. Cell Endocrinol.* 72:239-246.

Larrson, L.I., Fahrenkrug, J., Holst, J.J., Schaffalitzky de Muckadell, O.B. (1978) Innervation of the pancreas by vasoactive intestinal peptide (VIP) immunoreactive nerves. *Life Sci.* 22:773-780.

Larrson, L.I., Fahrenkrug, Schaffalitzky de Muckadell, O.B. (1977) Vasoactive intestinal polypeptide occurs in nerves of the female genitourinary tract. *Science* 197:1374-1375.

Levavi-Sivan, B. and Yaron, Z. (1992) Involvement of cAMP in the stimulation of GTH secretion from the pituitary of a teleost fish, tilapia. *Mol. Cell. Endocrinol.* 85:175-182.

Levavi-Sivan, B. and Yaron, Z. (1993) Intracellular mediation of GnRH action on GTH release in tilapia. *Fish Physiol. Biochem.* 11:1-6.

Lovejoy, D.A., Fischer, W.H., Ngamvongchon, S., Craig, A.G., Nahorniak, C.S., Peter, R.E., Rivier, J.E., Sherwood, N.M. (1992) Distinct sequence of gonadotropin releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution. *Proc. Nat'l. Acad. Sci. U.S.A.* 89:6373-6377.

Lovejoy, D.A., Fischer, W.H., Parker, D.B., McRory, J.E., Park, M., Lance, V., Swanson, P., Rivier, K.E., Sherwood, N.M. (1991) Primary structure of two forms of gonadotropin releasing hormone from brains of the American alligator (*Alligator mississippiensis*). *Regul. Pep.* 33:105-106.

McDonald J.K., Lumpkin, M.D., Samson, W.K., McCann, S.M. (1985) Neuropeptide Y affects secretion of luteinizing hormone and growth hormone in ovariectomized rats. *Proc. Natl. Acad. Sci. U.S.A.* 82:561-564.

Maeda, K., and Frohman, A. (1978). Dissociation of systemic and central effects of neurotensin on the secretion of growth hormone, prolactin, and thyrotropin,. *Endocrinology.* 103:1903-1909.

Magliulo-Cepriano, L. and Schreibman, M.P. (1993). The distribution of neuropeptide Y and dynorphin immunoreactivity in the brain and pituitary gland of the platyfish, *Xiphophorus maculatus*, from birth to sexual maturity. *Cell Tiss. Res.* 271:87-92.

Magliulo-Cepriano, L., Schreibman, M.P., Blüm, V. (1993). The distribution of immunoreactive FMRF-amide, neurotensin and galanin in the brain and pituitary gland of three species of *Xiphophorus* from birth to sexual maturity. *Gen. Comp. Endocrinol.* 92:269-280.

Magliulo-Cepriano, L., Schreibman, M.P., Blüm, V. (1994) Distribution of variant forms of gonadotropin-releasing hormone and beta gonadotropin I and II in *Xiphophorus* from birth to sexual maturity. *Gen. Comp. Endocrinol. accepted 1/20/94.*

Maiter, D., Hooi, S. C., Koenig, J. I., Martin, J. B. (1990). Galanin is a physiological regulator of spontaneous pulsatile secretion of growth hormone in the male rat. *Endocrinology.* 126:1216-1222.

Marchant, T.A., Chang, J.P., Nahorniak, C.S., Peter, R.E. (1989) Evidence that gonadotropin-releasing hormone also functions as a growth hormone-releasing factor in the goldfish. *Endocrinol.* 124:2509-2518.

Margolis-Kazan, H. and Schreibman, M.P. (1981). Cross-reactivity between human and fish pituitary hormones as demonstrated by immunocytochemistry. *Cell Tiss. Res.* 221:257-267.

Margolis-Nunno, H., Halpern-Sebold, L., Schreibman, M.P. (1986) Immunocytochemical changes in serotonin in the forebrain and pituitary of aging fish. *Neurobiol. Aging* 7:17-21.

Margolis-Kazan, H., Halpern-Sebold, L., Schreibman, M.P. (1985) Immunocytochemical localization od serotonin in the brain and pituitary gland of the platyfish, *Xiphophorus maculatus. Cell Tiss. Res.* 240:311-314.

Martinoli, M.G., Dubourg, P., Geffard, M., Calas, A., Kaj, O. (1990) Distribution of GABA-immunoreactive neurons in the forebrain of the goldfish. *Cell Tiss. Res.* 260:77-84.

Mason, A.J., Hayflick, J.S., Zoeller, R.T., Young III, W.S., Phillips, H.S., Nikolics, K., Seeburg, P.H. (1986) A deletion truncating the gonadotropin-releasing gene is responsible for hypogonadism in the *hpg* mouse. Science 234:1366-1371.

Matsutami, S., Uchiyama, H., Itoh, H. (1986) Cytoarchitecture, synaptic organization and fiber connections of the nucleus olfactoretinalis in a teleost *(Navadon modesto). Brain Res.* 373:126-138.

McCann, S. M., Vijayan, E., Koenig, J., and Krulich, L. (1982). The effects of neurotensin on anterior pituitary hormone secretion. *Ann. N.Y. Med. Sci.* 400:160-171.

Melander, T., Hokfelt, T., Rokaeus, A., Cuello, A. C., Oertel, W. H., Voerhofstad, A., Goldstein, M. (1986). Coexistence of galanin-like immunoreactivity with catecholamines, 5-hydroxytryptamine, GABA, and neuropeptides in the rat CNS. *J. Neurosci.* 6:3640-3654.

Merchenthaler, I. (1991) Current status of brain hypophysiotropic factors. Morphological aspects. *Trends Endocrinol. Metab.* 2:219-226.

Mikami S., Yamada, S., Hasegawa, Y., Miyamoto, K. (1988) Localization of avian LHRH-immunoreactive neurons in the hypothalmus of the domestic fowl, *Gallus domesticus*, and the Japanese quail, *Coturnix coturnix. Cell Tissue Res.* 251:51-58.

Miller, K.E. and Kriebel, R.M. (1986) Peptidergic innervation of caudal neurosecretory neurons. *Gen. Comp. Endocrinol.* 64:396-400.

Moons, L., Batten, T. F. C., Vandesande, F. (1991). Autoradiographic distribution of galanin binding sites in the brain and pituitary of the sea bass (Dicentrarchus labrax). *Neurosci. Lett.* 123:49-52.

Morris, Y.A. and Crews, D. (1990) The effects of exogenous neuropeptide Y on feeding and sexual behavior in the red-sided garter snake (*Thamnophis sirtalis parietalis*). *Brain Res.* 530:339-341.

Munz, H., Claas, B., Stumpf, W. E., Jennes, L. (1982). Centrifugal innervation of the retina by luteinizing hormone releasing hormone (LHRH)-immunoreactive telencephalic neurons in teleostean fishes. *Cell Tissue Res.* 222:313-323.

Münz, H., Stumpf, W.E., Jennes, L. (1981). LHRH systems in the brain of platyfish. *Brain Res.* 221:1-13.

Naito, N., Hyodo, S., Okumoto, N., Urano, A., Nakai, Y. (1991). Differential production and regulation of gonadotropins (GTH I and GTH II) in the pituitary gland of rainbow trout, *Oncorhynchus mykiss*, during ovarian development. *Cell Tissue Res.* 266:457-467.

Naito, N., Nakai, Y., Nagahama, Y., Suzuki, K., Kawauchi, H. (1988). Immunoelectron microscopy of two distinct gonadotrophs in teleost pituitary. *Zool. Sci.* 5:1302.

Ngamvongchon, S., Lovejoy, D.A., Fischer, W.H., Craig, A.G., Nahorniak, C.S., Peter, R.E., Rivier, J.E., Sherwood, N.M. (1992) Primary structures of two forms of gonadotropin-releasing hormone, one distinct and one conserved, from catfish brain. *Molec. Cell. Neurosci.* 3:17-22.

Noe, B.D., Milgram, S.L., Balasubramaniam, A., Andrews, P.C., Calka, J., McDonald. J.K. (1989) Localization and characterization of neuropeptide Y-like peptides in the

brain and islet organ of the anglerfish, *Lophius americanus*. *Cell Tissue Res.* 257:303-311.

Okuzawa, K., Amano, M., Kobayashi, M., Aida, K., Hanyu, I., Hasegawa, Y., Miyamoto, K. (1990). Differences in salmon GnRH and chicken GnRH-II contents in discrete brain areas of male and female rainbow trout according to age and stage of maturity. *Gen. Comp. Endocrinol.* 80:116-126.

Olivereau, M. (1978a) Serotonin and MSH secretion: Effect of parachlorophenylalanine on the pituitary cytology of the eel. *Cell Tiss. Res.* 191:83-92.

Olivereau, M. (1978b) Effect of parachlorophenylalanine, a brain serotonin depleter, on the prolactin cells of the eel pituitary. *Cell Tiss. Res.* 191:93-99.

Olivereau, M., and Rand-Weaver, M. (1994) Immunocytochemical study of the somatolactin cells in the pituitary of Pacific salmon, *Oncorhynchus nerka*, and *O. keta* at some stages of the reproductive cycle. *Gen. Comp. Endocrinol.* 93:28-35.

Olson, K.G. and Welch, S.P. (1991) The effects of dynorphin A (1-13) and U50, 488H on free intracellular calcium in guinea pig cerebellar synaptosomes. *Life Sci.* 48:575-581.

Page, I.H. (1976) The discovery of serotonin. *Perspect. Biol. Med.* 20:1-8.

Parker, S.L., Kalra, S.P., Crowley, W.R. (1991) Neuropeptide Y modulates the binding of a gonadotropin-releasing hormone (GnRH) analog to anterior pituitary GnRH receptor sites. *Endocrinology* 128:2309-2316.

Peng, C., Huang, Y.P., Peter, R.E. (1990) Neuropeptide Y stimulates growth hormone and gonadotropin release from the goldfish pituitary in vitro. *Neuroendocrinol.* 52:28-34.

Pernow, B., (1983) Substance P. *Pharmacol. Rev.* 35:85.

Peter, R.E. and Fryer, J.N. (1983) Endocrine functions of the hypothalmus of *Actinopterygians. In:* (Davis, R.E. and Northcutt, R.G. Eds.) Fish Neurobiology, Vol. 2. University of Michigan Press, Ann Arbor, pp. 165-201.

Peter, R.E. and Gill, V.E. (1975) A sterotaxic atlas and technique for forebrain nuclei of the goldfish, *Carassius auratus. J. Comp. Neurol.* 159:69-102.

Peter, R.E., Habibi, H.R., Chang, J.P., Nahorniah, C.S., Yu, K.L., Huang, Y.P., Marchant, T.A. (1990) Actions of gonadotropin releasing hormone (GnRH) in the

goldfish. *In*: (Epple, A., Scanes, C.G., Stetson, M.H. Eds.) Progress in Comparative Endocrinology. Wiley-Liss, N.Y. pp. 393-398.

Peter, R.E., Chang, J.P., Nahorniak, C.S., Omeljaniuk, R.J., Sokolowska, M., Shih, S.H., Billard, R. (1986) Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. *Recent Prog. Horm. Res.* 42:513-548.

Peter, R.E., Yu, K.L., Marchant, T.A., Rosenblum, P.M. (1990a) Direct neural regulation of the teleost adenohypophysis. *J. Expt. Zool.* 4:84-89.

Planas, J. V., Swanson, P., Rand-Weaver, M., Dickhoff, W. W. (1992). Somatolactin stimulates in vitro gonadal steriodogenesis in coho salmon, *Oncorhynchus kisutch. Gen. Comp. Endocrinol.* 1-5.

Polak, J. M., and Bloom, S. R. (1982). The central and peripheral distribution of neurotensin. *Ann. N.Y. Med. Sci.* 400:75-93.

Pontet, A., Danger, J.M., Dubourg, P., Pelletier, G., Vaudry, H., Kah, O. (1989) Distribution and characterization of neuropeptide Y-like immunoreactivity in the brain and pituitary of the goldfish. *Cell Tissue Res.* 255:529-538.

Powell, R.C., Jach, H., Millar, R.P., King, J.A. (1987) Identification of Gln^8-GnRH and His^5, Trp^7, Tyr^8-GnRH in the hypothalamus and extra-hypothalamic brain of ostrich (*Struthio camelus*). *Peptides.* 8:185-190.

Powell, R.C., Millar, R.P., King, J.A. (1986) Diverse molecular forms of gonadotropin-releasing hormone in an elasmobranch and a teleost fish. *Gen. Comp. Endocrinol.* 63:77-85.

Price, D. A., and Greenberg, M. J. (1977). Structure of a molluscan cardioexcitatory peptide. *Science.* 197:670-671.

Rama Krishna, N. S., Subhedar, N., Schriebman, M. P. (1992). FMRF-amide-like immunoreactive nervus terminalis innervation to the pituitary in the Catfish, *Clarias batrachus* (Linn.): Demonstration by lesion and immunocytochemical techniqes. *Gen. Comp. Endocrinol.* 85:111-117.

Rand-Weaver, M. and Swanson, P. (1993) Plasma somatolactin levels in coho salmon (*Oncorhynchus kisutch*) during smoltification and sexual maturation. *Fish Physiol. Biochem.* 11:175-182.

Rand-Weaver, M., Swanson, P., Kawauchi, H., and Dickhoff, W. W. (1992). Somatolactin, a novel pituitary protein: purification and plasma levels during reproductive maturation of coho salmon. *J. Endocrinol. In press.*

Reinecke, M. (1985). Neurotensin: immunohistochemical localization in central and peripheral nervous system and in endocrine cells and its functional role as a neurotransmitter and an endocrine hormone. *Prog. Histochem. Cytochem.* 16:1-72.

Rivier, C., Brown, M., Vale, W. (1977). Effects of neurotensin, substance P, and morphine sulfate on secretion of prolactin and growth hormone in the rat. *Endocrinology.* 751-754.

Rokaeus, A. (1987). Galanin: A newly isolated biologically active neuropeptide. *Trends in Neurosci.* 10:158-164.

Rokaeus, A., and Brownstein, M. J. (1986). Construction of a porcine adrenal medullary cDNA library and nucleotide sequence analysis of two clones encoding a galanin precursor. *Proc. Natl. Acad. Sci. USA* 83:6287-6291.

Rokaeus, A., Young III, W. S., Mezey, E. (1988). Galanin coexists with vasopressin in the normal rat hypothalamus and galanin's synthesis is increased in the Brattleboro (diabetes insipidus) rat. *Neurosci. Lett.* 90:45-50.

Sahu, A., Crowley, W.R., Kalra, S.P. (1990) An opioid-neuropeptide-Y transmission line to Luteinizing hormone (LH)-releasing hormone neurons: A role in the induction of LH surge. *Endocrinology* 126:876-883.

Said, S.I. and Mutt, V. (1970) Polypeptide with broad biological activity: isolation from small intestine. *Science* 169:1217-1218.

Sawyer, C.H. (1988) Anterior pituitary neural control concepts. *In:* (McCann, S.M., Ed) Endocrinology: People and Ideas. American Physiol. Soc., Baltimore, MD.

Schartl, M. (1990) Homology of melanoma-inducing loci in the genus *Xiphophorus*. *Genetics* 126:1083-1091.

Schartl, M. (1988) A sex chromosomal restriction-fragment-length marker linked to melanoma-determining *Tu* loci in *Xiphophorus*. *Genetics* 119:679-685.

Schartl, M. and Adams, D. (1992) Molecular cloning, structural characterization, and analysis of transcription of the melanoma oncogene of *Xiphophorus*. *Pig. Cell Res. Suppl.* 2:173-180.

Schreibman, M. P. (1986). The Pituitary Gland. *In* (P. K. T. Pang, and M. P. Schreibman, Eds.) Vertebrate Endocrinology: Fundamentals and Biomedical Implications. Vol. 1, pp. 11-55, Academic Press, New York.

Schreibman, M.P. and Kallman, K.D. (1977) The genetic control of the pituitary-gonad axis in the platyfish, *Xiphophorus maculatus. J. Exp. Zool.* 200:277-294.

Schreibman, M.P. and Margolis-Nunno, H. (1989). The brain-pituitary-gonad axis in poikilotherms. *In* (Schreibman, M.P. and Scanes, C.G., Eds.) Development, Maturation, and Senescence of Neuroendocrine Systems. Academic Press, New York.

Schreibman, M.P. and Margolis-Nunno, H. (1987). Reproductive biology of the terminal nerve (nucleus olfactoretinalis) and other LHRH pathways in teleost fishes. *Ann. N.Y. Acad. Sci.* 519:60-68.

Schreibman, M.P., Schartl, M., Kallman, K.D., Magliulo-Cepriano,L. (1994) Molecular approaches to the study of the genetic regulation of the fish reproductive system. *In*: Perspectives in Comparative Endocrinology. National Research Council, Canada, *in press.*

Schreibman, M. P., Holtzman, S., Cepriano, L. (1990). The life cycle of brain-pituitary-gonad axis in teleosts. *In* (Epple, A., Scanes, C.G., Stetson, M.H. Eds.) Progress in Comparative Endocrinology, Wiley-Liss, New York, pp 399-408.

Schreibman, M.P., Holtzman, S., Eckhardt, R.A. (1989) Genetic influences on reproductive system development and function: A reveiw. *Fish Physiol. Biochem.* 7:237-242.

Schreibman, M. P., Margolis-Nunno, H., Halpern-Sebold, L. (1987). Aging in the neuroendocrine system. *In* (Norris, D.O. and Jones, R.E., Eds.) Hormones and reproduction in fishes, amphibians, and reptiles. pp. 563-584, Plenum Press, New York.

Schreibman, M. P., Margolis-Nunno, H., Halpern-Sebold, L. (1986). The structural and functional relationships between olfactory and reproductive systems from birth to old age in fish. *In* (Duval, D., Muller-Schwarze, D. and Silverstein, R.M. Eds.) Chemical signals in vertebrates. Vol. 4, pp. 155-172, Plenum Press, New York.

Schreibman, M. P., Margolis-Kazan, H., Halpern-Sebold, L., O'Neill, P. A., Silverman, R. C. (1984). Structural and functional links between olfactory and reproductive systems:puberty-related changes in olfactory epithelium. *Brain. Res.* 302:180-183.

Schreibman, M. P., Halpern-Sebold, L. R., Ferin, M., Margolis-Kazan, H., and Th.Goos, H. J. (1983). The effect of hypophysectomy and gonadotropin administration on the distribution and quantity of LHRH in the brains of platyfish: A combined immunocytochemistry and radioimmunoassay study. *Brain. Res.* 267:293-300.

Schreibman, M. P., Margolis-Kazan, H., Halpern-Sebold, L., Goos, H. J. T. (1982). The functional significance of the nucleus olfactoretinalis in the platyfish *Xiphophorus maculatus*. *In* (Richter, C.J.J., and Goos, H.J.Th. Eds.) Proceedings of the Inter. Symp. on Reprod. Physiol. of Fishes. p. 59, Pudoc, Wacheningen.

Schreibman, M.P., Berkowitz, E.J., van der Hurk, R. (1982a) Histology and histochemistry of the platyfish *(Xiphophorus maculatus)* testis and ovary from birth to sexual maturity. Cell Tiss. Res. 224:81-87.

Schreibman, M.P., Pertschuk, L.P., Rainford, E.A., Margolis-Kazan, M., Gelber, S.J. (1982b) The histochemical localization of steroid binding sites in the pituitary gland of a teleost (the platyfish). *Cell Tiss. Res.* 226:523-530.

Schreibman, M.P., Halpern, L.R., Goos, H.J.Th., Margolis-Kazan, H. (1979) Identification of luteinizing hormone releasing hormone (LHRH) in the brain and pituitary gland of a fish by immunocytochemistry. *J. Exp. Zool.* 210:153-160.

Schreibman, M. P., Leatherland, J. F., McKeown, B. A. (1973). Functional morphology of the teleost pituitary gland. *Amer. Zool.* 13:719-742.

Schwanzel-Fekuda, M., Zheng, L.M., Bergen, H., Weesner, G., Pfaff, D.W. (1992) LHRH neurons: functions and development. *In:* (Swaab, D.F., Hofman, M.A., Mirmiran, M., David, R., van Leeuwen, F.W., Eds.) Progress in Brain Research. Elsevier Science Publishers B.V. pp. 189-203.

Seeburg, P.H. and Adelman, J.P. (1984) Characterization of cDNA for precursor of human luteinizing hormone-releasing hormone. *Nature* 311:666-668.

Sherwood, N. M. (1986). Evolution of a neuropeptide family: gonadotropin-releasing hormone. *Am. Zool.* 26:1041-1054.

Sherwood, N.M., Lovejoy, D.A., Coe, I.R. (1993) Origin of mammalian gonadotropin-releasing hormones. *Endocrine Rev.* 14:241-255.

Sherwood, N.M., Harvey, B., Brownstein, M.J., Eiden, L.E. (1984). Gonadotropin-releasing hormone in striped mullet (*Mugil cephalus*), milkfish (*Chanos chanos*) and rainbow trout(*Salmo gairdneri*), comparison with salmon GnRH. *Gen. Comp. Endocrinol.* 55:174-181.

Silverman, A.J., Jhamandas, J., Renaud, L.P. (1987) Localization of luteinizing hormone-releasing hormone (LHRH) neurons that project to the median eminence. *J. Neurosci.* 7:2312-2319.

Skofitsch, G., Jacobowitz, D. M., Amann, R., Lembeck, F. (1989). Galanin and vasopressin coexist in the rat hypothalamo-hypophyseal system. *Neuroendocrinol.* 49:419-427.

Sloley, B.D., Kah, O., Trudeau, V.L., Dulka, J.G., Peter, R.E. (1992) Amino acid neurotransmitters and dopamine in brain and pituitary of goldfish: Involvement in the regulation of gonadotropin secretion. *J. Neurochem.* 58:2254-2262.

Somoza, G.M., and Peter, R.E. (1991) Effects of serotonin on gonadotropin and growth hormone release from in vitro perifused goldfish pituitary fragments. *Gen. Comp. Endocrinol.* 82:103.

Somoza, G.M., Yu, K.L., Peter, R.E. (1988) Serotonin stimulates gonadotropin release in female and male goldfish, *Carassius auratus. Gen. Comp. Endocrinol.* 72:374-382.

Sower, S.A., Chiang, Y., Lovas, S., Conlon, J.M. (1993) Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain. *Endocrinol.* 132:1125-1131.

Stampinato, S., Canossa, M., Ventura, C., Bachetti, T., Venturini, R., Bastagli, L., Bernardi, P., Ferri, S. (1991) Heterogeneity of immunoreactive dynorphin B-like material in human, rat, rabbit and guinea pig heart. *Life Sci.* 48:551-559.

Stell, W. K., Walker, S. E., Ball, A. K. (1987). Functional-anatomical studies on the terminal nerve projections to the retina of bony fishes. *Ann. N.Y. Acad. Sci.* 80-96.

Stell, W. K., Chohan, K. S., Kyle, A. L. (1985). Substance P immunoreactivity coexists with LHRH and FRMFamide immunoreactivity in nervus terminalis efferents to the goldfish retina. *Invest. Opthol. Vis. Sci.* 277.

Stell, W. K., Walker, S. E., Chohan, K. S., Ball, A. K. (1984). The goldfish nervus terminalis: a luteinizing hormone-releasing hormone and molluscan cardioexcitatory peptide immunoreactive olfactoretinal pathway. *Proc. Natl. Acad. Sci.* 81:940-944.

Sternberger, L. A., Hardy, P. H., Cuculis, J. J., Meyer, H. G. (1970). The unlabelled antibody enzyme method of immunohistochemistry: preparation and properties of

soluble antigen-antibody complex (horseradish peroxidase) and its use in identification of spirochetes. *J. Histochem. Cytochem.* 18:315-333.

Stopa, E.G., Sower, S.A., Svendsen, C.N., King, J.C., (1988) Polygenic expression of gonadotropin releasing hormone (GnRH) in human? *Peptides*. 9:419-423.

Subhedar, N. and Rama Krishna, N.S., (1988). Immunocytochemical localization of LH-RH in the brain and pituitary gland of the catfish, *Clarias batrachus* (Linn.). *Gen. Comp. Endocrinol.* 72:431-442.

Sutton, S.W., Toyama, T.T., Otto, S., Plotsky, P.M. (1988) Evidence that neuropeptide Y (NPY) release into the hypophysial portal circulation participates in priming gonadotrophs to the effects of gonadotropin-releasing hormone (GnRH). Endocrinol. 123:1208-1210.

Suzuki, K., Kawauchi, H., Nagahama, Y. (1988). Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. *Gen. Comp. Endocrinol.* 71:292-301.

Suzuki, K., Nagahama, Y., Kawauchi, H., (1988a) Steroidogenic activities of two distinct salmon gonadotropins. *Gen. Comp. Endocrinol.* 71:452-458.

Swanson, P., Suzuki, K., Kawauchi, H., Dickhoff, W.W. (1991). Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. *Biol Reprod.* 44:29-38.

Swanson, P., Bernard, M., Nozaki, M., Suzuki, K., Kawauchi, H., Dickhoff, W.W. (1989) Gonadotropins I and II in juvenile coho salmon. *Fish Physiol. Biochem.* 7:169-176.

Swanson, P., Suzuki, K., Kawauchi, H. (1987) Isolation and biochemical characterization of two distinct pituitary gonadotropins from coho salmon *Oncorhynchus kisutch. Amer. Zool.* 27:79A.

Tanoh, T., Shimatsu, A., Murakami, Y., Ishikawa, Y., Yanaihara, N., Imura, H. (1991). Cholinergic modulation of growth hormone secretion induced by galanin in rats. *Neuroendocrinol.* 54:83-88.

Tatemoto, K., Rokaeus, A., Jornvall, H., McDonald, T. J., Mutt, V. (1983). Galanin-a novel biologically active peptide from porcine intestine. *FEBS* 164:124-128.

Tatemoto, K., Carlquist, M., Mutt, V. (1982) Neuropeptide Y: A novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature* 296:659.

Tillet, Y., Caldani, M., Batailler, M. (1989) Anatomical relationships of monoaminergic and neuropeptide Y-containing fibers with luteinizing hormone-releasing hormone systems in the preoptic area of the sheep brain: immunohistochemical studies. *J. Chem. Neuroanat.* 2:319-326.

Vallarino, M., Feuilloley, M., Vandesande, F., and Vaudry, H. (1991). Immunocytochemical mapping of galanin-like immunoreactivity in the brain of the dogfish *Scyliorhinus canicula. Peptides* 12:351-357.

Vijayan, E., and McCann, S. M. (1979). In vivo and in vitro effects of substance P and neurotensin on gonadotropin and prolactin release. *Endocrinology.* 105:64-68.

Woller, M.J. and Terasawa, E. (1991) Infusion of neuropeptide Y into the stalk-median eminence stimulates in vivo release of luteinizing hormone-releasing hormone in gonadectomized Rhesus monkeys. *Endocrinol.* 128:1144-1150.

Wong, A.O.L., Chang, J.P., Peter, R.E. (1993) Dompamine functions as a growth hormone-releasing factor in the goldfish, *Carassius auratus. Fish Physiol. Biochem.* 11:77-84.

Wright, D.E. and Demski, L.S. (1991) Gonadotropin hormone-releasing hormone (GnRH) immunoreactivity in the mesencephalon of sharks and rays. *J. Comp. Neurol.* 307:49-56.

Yaron, Z. and Levavi-Sivan, B. (1990) Intracellular events associated with GnRH and dopamine effects in tilapia. *In:* (Epple, A., Scanes, C.G., Stetson, M.H., Eds.) Progess in Comparative Endocrinology. Wiley-Liss, New York 342:409-414.

Yu, K.L., Rosenblum, P.M., Peter, R.E. (1991). *In vitro* release of gonadotropin-releasing hormone from the brain preoptic-anterior hypothalmic region and pituitary of female goldfish. *Gen. Comp. Endocrinol.* 81:26-267.

Yu, K.L., Sherwood, N.M., Peter, R.E. (1988). Differential distribution of two molecular forms of gonadotropin-releasing hormone in discrete brain areas of goldfish (*Carassius auratus*). *Peptides*. 9:625-630.

Zambrano, D. (1972) Innervation of the teleost pituitary. *Gen. Comp. Endocrinol.* 3:22-31.