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UNIVERSITY OF ALBERTA

REGULATION OF GONADOTROPIN-RELEASING HORMONE IN GOLDFISH BRAIN

by

KEI-LI YU

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

SPRING, 1990

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ABSTRACT

This study concerned the regulation of gonadotropin-releasing hormone (GnRH) in goldfish brain. A radioimmunoassay (RIA) for [Trp^7, Leu^8]-GnRH was validated. Marked decreases in GnRH levels in discrete brain areas and the pituitary were associated with the ovulatory gonadotropin (GtH) surge. A similar negative correlation between GnRH levels in discrete forebrain areas and the pituitary, and serum GtH levels was also observed in male goldfish during spawning with spontaneously ovulating females. The simultaneous changes in GnRH concentrations in discrete forebrain areas suggested that the GnRH neuronal system may function as an integrated unit for the activation of GtH secretion in goldfish during spawning.

The centrally active dopaminergic antagonist pimozide caused time and dose dependent increases in serum GtH levels and accumulation of GnRH in the olfactory bulbs, telencephalon and pituitary, through an apomorphine-sensitive dopaminergic mechanism. Male goldfish exposed to prostaglandin (PG)-treated females for one or two hours had significantly increased serum GtH levels and GnRH concentrations in discrete forebrain areas. These GtH and GnRH responses were abolished by sectioning of the olfactory tracts (OT), specifically the medial OT in the male goldfish. Pretreatment of male goldfish with pimozide potentiated the increases in serum GtH levels and caused a marked reduction of GnRH in the olfactory bulbs, telencephalon and pituitary in response to PG-treated females. The results together suggest that the central dopaminergic system has an inhibitory influence on the brain GnRH system and pituitary GtH secretion in normal and behaviorally-stimulated male goldfish.

Using an *in vitro* static incubation system, norepinephrine was found to stimulate GnRH release from slices of the preoptic-anterior hypothalamus through the α_1-adrenergic

receptors. The inhibitory effects of dopamine on GnRH release from preoptic-anterior hypothalamic slices and pituitary fragments are mediated by D_1- and D_2- receptors, respectively. Serotonin stimulates GnRH release from both tissue preparations.

Using a combined RIA and high performance liquid chromatography approach, the two molecular forms of GnRH in goldfish brain and pituitary were characterized to be [Trp^7, Leu^8]-GnRH and [His^5, Trp^7, Tyr^8]-GnRH; the two forms of GnRH have a differential distribution. The relative functional roles of the two GnRH molecular forms remain a challenging area to be studied in the future.

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1. GENERAL INTRODUCTION

It is generally accepted in teleosts as in other vertebrates that reproductive activity is controlled by the brain in response to neural information about physiological and environmental conditions (see Ball, 1981; Shivers *et al*, 1983). The concept that the central nervous system controls the secretion of anterior pituitary hormones through release of chemical factors from the hypothalamus was proposed more than 40 years ago (Harris, 1955). This concept led to the isolation and characterization of several hypothalamic-hypophysiotrophic factors, known as releasing factors in mammals (Blackwell and Guillemin, 1973). The key hypothalamic releasing factor or brain peptide that stimulates the secretion of the gonadotropin (GtH) hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), was first isolated from porcine (Matsuo *et al.*, 1971) and ovine (Burgus *et al.*, 1972) hypothalami. This decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) called gonadotropin-releasing hormone (GnRH), or luteinizing hormone-releasing hormone (LHRH), was later found in hypothalamic tissues of other mammals and in human placental tissues (see Seeburg *et al.*, 1987).

In teleosts and other nonmammalian vertebrates, the existence of multiple molecular GnRH forms in the brain of a single species has been well demonstrated (see Millar and King, 1987; Sherwood, 1986, 1987). Research over the past nine years has led to the isolation and full characterization of four molecular forms of GnRH in nonmammalian vertebrates. Two GnRHs have been isolated from the chicken hypothalami: [Gln^8]-GnRH (chicken GnRH-I, cGnRH-I; King and Millar, 1982a,b) and [His^5, Trp^7, Tyr^8]-GnRH (chicken GnRH-II, cGnRH-II; Miyamoto *et al.*, 1984). [Trp^7, Leu^8]-GnRH was isolated from salmon brain (Sherwood *et al.*, 1983) and [Tyr^3, Leu^5, Glu^6, Trp^7, Lys^8]-GnRH from lamprey brain (Sherwood *et al.*, 1986). The presence in brains of higher mammals

of other GnRH forms or structurally homologous peptides, other than LHRH, is controversial (King *et al.*, 1988; Stopa *et al.*, 1988); however, the existence of a second form similar to cGnRH-II in the brain of some marsupial mammals has been reported recently (Millar and King, 1988).

The general paucity of information on the physiology of brain GnRH neuronal system in teleosts is due, in part, to the lack of a sensitive validated assay system for the piscine form of GnRH. Most of our understanding of the brain GnRH neuronal activity in teleosts was derived indirectly from experiments which relied on measurement of pituitary GtH secretion or sex steroid production. The elucidation of the structure and synthesis of a piscine GnRH peptide, sGnRH, made possible more direct study of the physiology of brain GnRH in teleosts. In this thesis, a radioimmunoassay (RIA) for sGnRH was developed to directly measure the GnRH content in discrete brain areas and the pituitary, to study the relationship between brain GnRH and serum GtH levels during different physiological conditions, and to investigate the possible role of dopamine, and other monoamines, in the regulation of brain GnRH levels and release in goldfish.

In view of the rapid growth of literature concerning the physiology of the brain GnRH neuronal system and its regulation in mammals, the following sections review first the current literature in this area from studies in mammals and other lower vertebrates (section 1.1), and then teleosts (section 1.2). This provides a background for the present study on the regulation of brain GnRH activity in goldfish.

1.1 GnRH neuronal system and its regulation in mammals and lower vertebrates.

1.1.1 Localization of GnRH in brain

In mammals and in other vertebrates, immunoreactive GnRH (ir-GnRH) cell bodies were found in a continuum starting from the olfactory bulbs region associated with the nervus terminalis (terminal nerve, TN), through the ventromedial telencephalon, medial

septal area and suprachiasmatic region to the medial preoptic areas and the medial basal hypothalamus (MBH) (see Barry, 1979; Peter, 1986; Silverman, 1988). Axons of GnRH neurons in the medial preoptic area and, possibly some axons from neurons located outside these areas, project particularly to the external layer of the anterior median eminence (ME) in the rat (Anthony *et al.*, 1984; King and Anthony, 1984), although other sites of innervation in the pituitary have been reported in other mammalian species (Anthony *et al.*, 1984). The ir-GnRH axons also project to the organum vasculosum lamina terminalis (OVLT) where a high concentration of GnRH immunoactivity can be detected (see Barry, 1979; Silverman, 1988). Moreover, ir-GnRH axons can be detected in many regions of the brain, including the midbrain central gray (Samson *et al.*, 1980; King *et al.*, 1984); GnRH release in this region has been implicated in facilitating lordosis behavior in rats (see Pfaff and Schwartz-Giblin, 1988). This wide distribution of GnRH in the brain and the demonstration of GnRH receptors in different brain regions (Reubi and Maurer, 1984; Badr and Pelletier, 1987; Jennes *et al.*, 1988; Leblanc *et al.*, 1988) suggests that GnRH may also play a neuromodulator and/or neurotransmitter role in the central nervous system.

In rats, the preoptic nucleus-suprachiasmatic region and the arcuate nucleus in the MBH have been proposed as the two hypothalamic centers responsible for cyclic (preovulatory surge) and tonic (episodic) release of GtH respectively (see Kalra and Kalra, 1983). In primates (see Pohl and Knobil, 1982; Knobil and Hotchkiss, 1988), it appears that major GnRH pulse-generating circuits for both tonic and surge release of GtH are located within the MBH (specifically arcuate nucleus) and are capable of functioning independent of innervation from the other parts of the brain.

The distribution of ir-GnRH cell bodies and fibers in the brain of nonmammalian vertebrates shows a pattern similar to that described in mammals (see Peter, 1986; Silverman, 1988). However, the distribution of different forms of GnRH has not been addressed in most studies. Using antisera raised against cGnRH-I and cGnRH-II

respectively, a differential distribution of the two chicken GnRH forms was found in the brains of chicken and Japanese quail (Hasegawa *et al.*, 1986; Mikami *et al.*, 1988). These studies showed that cGnRH-I, but not cGnRH-II immunoactivity is present in the ME and ir-cGnRH-I cell bodies are located in the preoptic-septal region; whereas, ir-cGnRH-II neurons are found only in the midbrain of chicken and Japanese quail (Hasegawa *et al.*, 1986; Mikami *et al.*, 1988), suggesting differential functions of these two GnRH forms in avian species. Using an antiserum against sGnRH, one of the GnRH forms in reptiles, a recent immunocytochemical study on chameleon also localized ir–GnRH cell bodies in the anterior midbrain tegmentum, but not in hypothalamic areas (Bennis *et al.*, 1989). The immunocytochemical distribution of the different forms of GnRH in teleosts has not been investigated.

1.1.2 Biosynthesis and processing of GnRH precursor.

GnRH, in common with other small neuropeptides, is synthesized as part of a larger precursor in rat brain, human brain and placenta (see Seeburg *et al.*, 1987). This GnRH preprohormone, made up of 92 amino acids in humans and rats is comprised of a signal sequence of 23 amino acids followed by the decapeptide, a Gly-Lys-Arg sequence for enzymatic processing, and a 56 amino acid C-terminal sequence (see Nikolics, 1989). The 56 amino acid C-terminal sequence of the precursor constitutes the GnRH-associated peptide (GAP). The sequence of GAP is different among human, rat and mouse (see Nikolics, 1989). GAP has been shown to have prolactin-releasing inhibiting activity in rats (Nikolics *et al.*, 1985; Yu *et al.*, 1988), but not in sheep (Thomas *et al.*, 1988). Although further postranslational processing of GAP into smaller peptides has been suggested (Millar *et al.*, 1986; Silverman *et al.*, 1987; Ackland *et al.*, 1988; Wetsel *et al.*, 1988), secretion of GnRH and GAP simultaneously from the ME *in vitro* (Valenca *et al.*, 1988) and into portal blood *in vivo* (Clarke *et al.*, 1987) has been reported. Postranslational processing of GnRH-GAP prohormone in neuronal cell bodies and during

axonal transport seems to vary among different species (see King *et al.*, 1985). In the primate brain, GnRH-GAP cleavage begins and reaches completion within cell bodies before axonal transport of the products (King *et al.*, 1985; Ronnekleiv *et al.*, 1987). In rats, cleavage of the GnRH decapeptide from the larger precursor occurs primarily in the distal portion of the neuronal fibers (King and Anthony, 1983). Analysis of genes for multiple GnRH forms in nonmammalian vertebrates has not yet been reported although immunoactivity for the human GAP sequence has been demonstrated in amphibia (Andersen *et al.*, 1988).

1.1.3 Pulsatile release of GnRH

In mammals, it has been proposed that ultradian fluctuations of serum LH are a consequence of pulsatile secretion of LH from the pituitary in response to the pulsatile release of GnRH (see Lincoln, 1988). The majority of the information on GnRH secretion supporting this idea had been deduced indirectly from (i) the sequential measurement of circulating LH levels under differing physiological and experimental conditions (see Lincoln, 1988; Robinson and Dyer, 1988); (ii) changes in LH secretion following the administration of GnRH or its analogs (see Knobil, 1980; Karsch *et al.*, 1984), or (iii) the removal of endogeneous GnRH by passive and active immunization (Arimura *et al.*, 1976; McCormack *et al.*, 1977; Lincoln and Fraser, 1979; Ellis *et al.*, 1983). By push-pull perfusion of the ME (rat: Levine and Ramirez, 1982), or sampling of the hypophyseal portal blood (rhesus monkey: Carmel *et al.*, 1976; sheep: Clarke and Cummins, 1982; Levine *et al.*, 1982), simultaneous measurements of GnRH and LH in conscious animals have been possible. Results from these studies confirm that GnRH release is pulsatile and is closely correlated with LH pulses, strongly supporting the view that GnRH pulses regulate pulsatile release of pituitary LH. Temporal association between GnRH and FSH release has also been shown in the mare (Alexander and Irvine, 1987) and in

orchidectomized rats (Levine and Duffy, 1988); but not in short-term castrated rats (Urbanski *et al.*, 1988).

1.1.4 Enzymatic degradation of GnRH.

GnRH released into the hypophyseal portal blood vessels is removed at the pituitary by receptor binding and internalization within gonadotrophs (see Conn *et al.*, 1987) or by enzymatic degradation (see Griffiths and McDermott, 1983). Several degrading enzymatic activities have been demonstrated in the hypothalamus and pituitary in mammals (see McKelvy and Blumberg, 1986), bird (hens: Advis *et al.*, 1985) and fish (gilthead seabream: Zohar *et al.*, 1989). GnRH peptidase activity in discrete hypothalamic regions and the anterior pituitary varies during the estrous cycle in female rats, and is inversely correlated with the ME GnRH content, suggesting that degradation of GnRH released from the ME may be regulated by GnRH peptidase activities which are in turn under regulatory control by gonadal steroids (Griffiths and McDermott, 1983). Recently, the principal enzyme involved in the degradation of the Tyr^5-Gly^6 bond of GnRH in both hypothalamic and pituitary membranes has been characterized to be the metalloendopeptidase-24.15 (Molineaux *et al.*, 1988).

1.1.5 Regulation of GnRH neuronal activity during gonadal cycles.

There is evidence to suggest that hypothalamic GnRH neuronal activity is regulated during gonadal cycles in birds (Knight *et al.*, 1984; Johnson and Advis, 1985) and in mammals (see Kalra and Kalra, 1983). In rats, detailed accounts of the changes in GnRH content of various hypothalamic nuclei during estrous cycles have been presented. GnRH content of the suprachiasmatic nucleus, preoptic nucleus, retrochiasmatic area and ME increase in the morning of proestrus and decrease in the afternoon at the time of the LH surge (Rance *et al.*, 1981; Wise *et al.*, 1981). A recent study on GnRH mRNA levels

suggests that GnRH synthesis is low in the morning of proestrus, but high in the afternoon when GnRH is released (Zoeller and Young, 1988), suggesting simultaneous activation of both GnRH synthesis and secretion at the time of the LH surge. Preovulatory surges of GnRH have been detected in the hypophyseal portal blood in rats, sheep and rhesus monkey, and in circulating blood of women (see Lincoln, 1988). These observations and results from other studies led to the suggestion that surges of GnRH secretion, together with marked increases in responsiveness of the pituitary to GnRH, ultimately trigger the preovulatory surge release of LH (see Fink, 1988). In contrast, a permissive role of GnRH in causing LH release has been suggested for the rhesus monkey (see Knobil, 1980; Knobil and Hotchkiss, 1988). In rhesus monkeys in which in the arcuate nucleus was lesioned, it was found that hourly pulses of GnRH at a constant dosage restored the complete hormonal (estrogen, progestrogen, LH and FSH) profile of the normal menstrual cycle (Knobil, 1980, Wildt *et al.*, 1981).

1.1.6 Behavioral or pheromonal influence on GnRH neuronal activity.

Behavioral or pheromonal regulation of GnRH neuronal activity has been implicated in studies of mammals as well as in other vertebrate species (see Harding, 1981; Allen and Adler, 1985; Crews and Silver, 1985; Sachs and Meisel, 1988). In mammals, GnRH is also known to facilitate reproductive behavior in addition to its hypophysiotrophic effects on GtH secretion (see Moss *et al.*, 1988; Pfaff and Schwartz-Giblin, 1988). Changes in GnRH neuronal activity in response to behavioral or pheromonal stimuli have been demonstrated. In female newts, GnRH content in the anterior telencephalon was elevated during sexual behavior with males (Moore *et al.*, 1987). In male mice, exposure to other males or ovariectomized females resulted in an increase in GnRH concentrations in the olfactory bulbs (Dluzen and Ramirez, 1983). In the female prairie vole, GnRH levels in the olfactory bulbs increased following exposure to male urine (Dluzen and Ramirez, 1981). In short-tailed field voles, the copulation induced LH surge and reflex ovulation are

associated with a marked drop in hypothalamic GnRH content (Versi *et al.*, 1982). A preovulatory rise in hypophyseal blood levels of GnRH was found in rabbits during the LH surge and ovulation induced by cupric acetate treatment (Tsou *et al.*, 1977). These studies suggest that hypothalamic GnRH neurons may be the common neurochemical pathway mediating the preovulatory LH surge in spontaneous and reflex ovulators (see Ramirez and Beyer, 1988).

1.1.7 Regulation of GnRH neuronal activity by gonadal steroids.

Results from various studies, mostly on rats, suggest that GnRH neuronal activity is regulated by the feedback of gonadal steroids, by central release of various neurotransmitters (see Kalra and Kalra, 1983; Goodman, 1988; Weiner *et al.*, 1988), and by short-loop feedback from GtH (Turgeon and Barraclough, 1976; Melrose, 1987) and GnRH (DePaolo *et al.*, 1987; Sarkar, 1987; Valenca *et al.*, 1987b; Zanisi *et al.*, 1987; Hiruma *et al*, 1989). Direct synapses between GnRH neurons in preoptic region (rat: Leranth *et al.*, 1985b; Witkin and Silverman, 1985; Pelletier, 1987; rhesus monkey: Thind and Goldsmith, 1988) or membrane appositions between GnRH axons in the ME (golden hamster: Lehman and Silverman, 1988; sheep: Lehman *et al.*, 1988) have been demonstrated in mammals.

In mammals, both positive and negative feedbacks of gonadal steroids on GtH secretion have been suggested to occur in females (see Kalra and Kalra, 1983; Fink, 1988; Goodman, 1988), whereas the control of tonic GtH secretion in males operates primarily by negative feedback of steroids (see Kalra and Kalra, 1983; Fink, 1988). In higher primates, evidence suggests that negative feedback effects of steroids on GtH secretion only operates in males (see Knobil and Hotchkiss, 1988). The exact nature of the changes in GnRH neuronal activity under different steroid feedback conditions has been studied only recently.

Negative steroid feedback. There is strong evidence that gonadectomy decreased hypothalamic GnRH levels in rats (see Kalra and Kalra, 1983, Kalra, 1985) and sheep (Wheaton, 1979), but increased the levels in cockerels (Knight *et al.*, 1983) . On the basis of measurements of the temporal changes in GnRH content of the hypothalamus and LH release in castrated male rats, Kalra (1985) suggested that changes in the GnRH content of MBH and ME after castration and testosterone replacement were not simply a result of altered secretion, but may be due to alteration in GnRH synthesis, prohormone processing and degradation. In support of this notion, recent studies using nucleotide probes for the GnRH prohormone indicate that gonadectomy decreases and gonadal steroid treatment increases the GnRH mRNA levels in rats (Pfaff, 1987; Rothfeld *et al.*, 1987; Roberts *et al.*, 1989). Synthesis and processing of the GnRH prohormone-like immunoactivity is also decreased after castration (Culler *et al.*, 1988). These results suggest that changes in GnRH synthesis and secretion after gonadectomy are regulated by dissociable mechanisms.

Recent studies showed that the frequency, but not amplitude, of pulsatile GnRH release was increased in short-term castrated rats, suggesting GnRH pulse frequency may play a role in the gonadal negative feedback suppression of LH (Levine and Duffy, 1988). In the ovariectomized (OVX) ewe, pulsatile secretion of GnRH into portal blood was inhibited by long term implants of estradiol (Karsch *et al.*, 1987), but was unaffected by acute estradiol treatment (Clarke and Cummins, 1985) . In long term OVX rats (Sarkar and Fink, 1980), but not in rhesus monkeys (Carmel *et al.*, 1976), acute treatment with estradiol reduced GnRH levels in hypophyseal portal blood. Thus, the negative feedback effects of steroids on GnRH secretion seem to be dose and/or time-dependent and may vary with species.

In contrast, other studies suggest that hypersecretion of GnRH may not be the sole determinant of the increase in LH secretion after gonadectomy (see Kalra, 1986). GnRH release into hypophyseal portal blood was decreased after orchidectomy in rats and was

restored to normal levels with testosterone replacement (Ching *et al.*, 1987). *In vitro* GnRH release from the hypothalamus (Rudenstein *et al.*, 1979; Dluzen and Ramirez, 1986; Nikolarakis *et al.*, 1986b; Kalra *et al.*, 1987) and ME (Valenca *et al.*, 1987a) was also lower in gonadectomized rats compared to normal rats.

Positive steroid feedback. A positive feedback effect of estrogen on GnRH secretion *in vivo* has been shown in long-term OVX rats (Sarkar and Fink, 1979, 1980), long-term OVX ewes (Clarke and Cummins, 1985; Schillo *et al.*, 1985), seasonally anoestrous ewes (see Clarke, 1988), OVX monkeys (Levine *et al.*, 1985), castrated male monkeys (Pau *et al.*, 1988) and women in mid-follicular phase of the menstrual cycle (Miyake *et al.*, 1983). Ohtsuka *et al* (1989) showed that estradiol induced release of GnRH from MBH of female rats *in vitro* while others showed that estradiol has minimal effects (Drouva *et al.*, 1985). Estradiol has also been found to facilitate GnRH release by increasing the size of the readily releasable pool of GnRH (Dyer *et al.*, 1980) and by stimulating the processing of GnRH from its precursor (Drouva *et al.*, 1986).

Growing evidence suggests that progesterone has a facilitatory role in the preovulatory surge of LH in rats. *In vitro* GnRH release from hypothalami was enhanced in progesterone-treated estrogen-primed OVX (OVX-EP) rats compared to estrogen-treated OVX (OVX-E) rats (Ramirez *et al.*, 1985). Progesterone, and its metabolites 5β, 3β-pregnanolone also enhanced *in vitro* and *in vivo* GnRH release from hypothalami in both OVX-E rats and in cycling female rats at proestrus (Ramirez *et al.*, 1985; Kim and Ramirez, 1986; Park and Ramirez, 1987). Other studies showed that treatment in the morning of OVX-E rats with progesterone induces an increase in GnRH content of MBH similar to that observed in the morning of proestrus in cyclic females, whereas OVX-E rats showed a decline in hypothalamic GnRH content during the same time period (see Kalra and Kalra, 1983; Peduto and Mahesh, 1985). In rabbits, progesterone increases GnRH release from push-pull perfused hypothalami in females, but not in males (Lin and

Ramirez, 1988). In domestic hen, passive immunoneutralization of GnRH blocks the positive feedback action of progesterone on LH secretion (Fraser and Sharp, 1978). In contrast, progesterone is inhibitory to the preovulatory LH surge and has been shown to decrease pulsatile GnRH secretion into the hypophyseal portal blood in ewes (Karsch *et al.*, 1987).

Sites of steroid action. Although studies in mammals, particularly in rats, clearly demonstrate that hypothalamic GnRH neuronal activities (GnRH synthesis, processing, release and degradation) are under the influence of gonadal signals (see Fink, 1988), GnRH neurons may not be the target of steroids. Nuclear estradiol (rat: Shivers *et al.*, 1983) or progesterone (hen: Sterling *et al.*, 1984; rat: Fox *et al.*, 1986) receptors are not found in the cell bodies of GnRH neurons. However, catecholaminergic neurons in the brain stem, dopamine (DA) neurons in the arcuate nucleus, endogenous opiod peptides (EOP) and gamma-aminobutyric acid (GABA)-containing neurons in the hypothalamic area are able to express estrogen receptors (see Pfaff and Schwartz-Giblin, 1988). Analysis at the light microscopic level has suggested association between GnRH neurons (or their processes) and neurons containing tyrosine-hydroxylase, dopamine β-hydroxylase, serotonin (5-HT), glutamic acid decarboxylase, neurotensin and substance P (see Silverman, 1988). In rats, β-endorph. (β-END; Chen *et al.*, 1989), ^{3}H-labeled catecholamine (Watanabe and Nakai, 1987), GABA (Leranth *et al.*, 1985a, 1988) and serotonin containing nerve terminals (Kiss and Halasz, 1985) directly synapse with GnRH neurons in the preoptic region. Other ultrastructural studies showed that axons containing opiod peptides in the arcuate nucleus-infundibular region of juvenile monkeys (Thind and Goldsmith, 1988), and DA-containing nerve terminals in ME of ewes (Kuljis and Advis, 1989) also make direct synaptic contact with GnRH-containing neurons. These results suggest that gonadal steroids regulate GnRH secretion indirectly, probably by altering the synthesis and release of other neurotransmitters; for example, effects of steroids on catecholamine turnover in hypothalamus of OVX rats have been shown (Wise *et al.*,

1981). Nevertheless, rapid *in vitro* effects of ovarian hormones on the release of GnRH indicates that ovarian hormones may also act directly through nongenomic actions, perhaps involving the cell membrane-mediated mechanisms (Drouva *et al.*, 1985; Ramirez *et al.*, 1985; Melrose and Gross, 1987).

1.1.8 Regulation of GnRH neuronal activity by monoamines.

The involvement of monoamines in the control of GnRH neuronal activity in mammals has been implicated using several physiological and experimental models (see Barraclough and Wise, 1982; Ramirez *et al.*, 1984; Weiner *et al.*, 1988).

Dopamine . Evidence exists to suggest both stimulatory and/or inhibitory roles of DA in the regulation of GnRH secretion in mammals (see Barraclough and Wise, 1982; Ramirez *et al.*, 1984; Freeman, 1988; Weiner *et al.*, 1988). The conflicting findings in the literature on the role of DA in the regulation of LH secretion in rats has been explained by the existence of multiple dopaminergic receptor subtypes for DA (Sarkar and Fink, 1981) and multiple dopaminergic pathways (Mackenzie *et al.*, 1984; James *et al.*, 1987) which may control LH release in opposing manners. The actions of DA on GnRH release from hypothalami *in vitro* are also conflicting. Stimulatory effects of DA on GnRH release from MBH (Jarjour *et al.*, 1986) and ME (Negro-Vilar *et al.*, 1979) in intact, but not castrated male rats have been found.

Norepinephrine. Neurons containing norepinephrine (NE) have long been proposed as a stimulatory component of the GnRH pulse generator (see Barraclough and Wise, 1982). Central administration of NE stimulates both GnRH and LH secretion in intact or OVX-E rabbits (Pau and Spies, 1986; Ramirez *et al.*, 1986), OVX-EP rats (Ching and Krieg, 1986), and in OVX rhesus monkeys (Terasawa *et al.*, 1988). In the rhesus monkey, intraventricular (i.v.) administration of α-adrenergic receptor blockers dampened or abolished GnRH pulsatile release (Terasawa *et al.*, 1988; Pau *et al.*, 1989).

Synchrony between NE and GnRH pulses has been observed during push-pull perfusion of the ME in rhesus monkeys (Terasawa *et al.*, 1988), but not between NE and LH released during push-pull perfusion of MBH in OVX-E rats (Jarry *et al.*, 1986). In contrast, inhibitory actions of NE or its agonists on GnRH release *in vivo* from OVX rabbit hypothalamus (Pau and Spies, 1986) and on pulsatile LH release in OVX rats (Gallo and Drouva, 1979; Gallo, 1984) and intact anoestrous ewes (Goodman, 1989) have been demonstrated. *In vitro* studies showed that GnRH release from ME can be stimulated by NE (Negro-Vilar *et al.*, 1979; Ojeda *et al.*, 1982). Stimulation of GnRH release from ME *in vitro* by NE has been shown to be mediated by α_1- (Heauline and Dray, 1984) or α_2-adrenergic receptors (Negro-Vilar, 1982); however, stimulation of GnRH/LH release *in vivo* by NE involves an α_1 receptor subtype (Coen and Coombs, 1983; Terasawa *et al.*, 1988).

It is also generally accepted that NE is also important in generation of the preovulatory GnRH surge in mammals (see Barraclough and Wise, 1982; Ramirez *et al.*, 1984). A noradrenergic pathway, originating primarily from the mesencephalic A1 and A2 cell groups, and projecting through the ventral noradrenergic tract onto the GnRH neurons in the preoptic area has been shown to be important for stimulating GtH secretion (see Barraclough *et al.*, 1984; Ramirez *et al.*, 1984). By measuring catecholamine turnover and GnRH content in discrete hypothalamic areas in rats, an increase in NE turnover rate at the time of the GnRH and LH surges on the afternoon of proestrus in rats was observed (Rance *et al.*, 1981). These results are in close agreement with the changes in the NE turnover rate during LH surges in OVX-EP rats (Wise *et al.*, 1981), supporting the view that NE may mediate the positive feedback actions of steroids. Moreover, an increase in NE released from the push-pull perfused medial preoptic area also occurs during the LH surge in OVX-E rats (Demling *et al.*, 1985).

Epinephrine. There is evidence to suggest involvement of epinephrine (E)-

containing neurons in the preovulatory GnRH surge. Selective pharmacological suppression of E synthesis delayed the accumulation of GnRH in the ME, and the LH surge, in OVX-E-P rat (Alder *et al.*, 1983). The turnover rate of hypothalamic E has been reported to increase on the afternoon of proestrus in cyclic rats (Coombs and Coen, 1983) or during LH surges in OVX-EP rats (Alder *et al.*, 1983), but not in another similar study in rats (Rance *et al.*, 1981). Kalra (1986) hypothesized that NE stimulates accumulation of GnRH in the ME, and E stimulates release of GnRH into the portal circulation.

Serotonin. The role of 5-HT in the control of the LH surge is controversial (see Freeman, 1988; Weiner *et al.*, 1988). Charli *et al* (1978) found that 5-HT inhibits release of GnRH from MBH. In contrast, it was shown that 5-HT can stimulate GnRH release during a double chamber perifusion of the ME and pituitary from proestrus rats (Vitale *et al.*, 1986). In another *in vitro* study, 5-HT also increased the frequency of release from hypothalami obtained from OVX-E rats, but had no effects on hypothalami from OVX rats (Meyer, 1989), suggesting a dependency of the 5-HT actions on sex steroids.

1.1.9 Regulation of GnRH neuronal activity by neuropeptides.

Neuropeptide Y. Simultaneous changes of both neuropeptide Y (NPY) and GnRH concentrations in the ME have been described during the LH surge in OVX-EP rats (Crowley *et al.*, 1985), and immunoneutralization of NPY inhibits the steroid induced LH surge in OVX rats (Wehrenberg *et al.*, 1989). These results suggest that NPY may participate in the neural regulation of GnRH and LH secretion. Recent studies showed that NPY stimulates the *in vitro* release of GnRH from the MBH in OVX-E rats (Sabatino *et al.*, 1989) or OVX-EP rats but not from untreated OVX rats (Crowley and Kalra, 1987). In other studies, NPY increases GnRH release during push-pull perfusion of the ME in intact conscious rabbits, but decreased release of GnRH in castrated animals (Khorram *et al.*, 1987). These and other observations suggest that NPY and NE actions on GnRH neurons, may be important in triggering the preovulatory and ovarian steroid-induced LH

surges in rats (see Kalra *et al.*, 1988)

Opiod peptides. In recent years, considerable evidence indicates that endogenous and exogenous opiates inhibit both tonic and surge release of GnRH and LH in mammals (see Ferin *et al.*, 1984; Kalra and Kalra, 1984) and in birds (Stansfield and Cunningham, 1987a,b, 1988). Morphine and EOP block the proestrus surge of GnRH and LH in rats (Ching, 1983). EOP can also inhibit the depolarization-induced release of GnRH from MBH-ME *in vitro* in intact male rats (Drouva *et al.*, 1980, 1981), but not in castrated rats (Nikolarakis *et al.*, 1986b). Administration of naloxone, an opiate receptor antagonist, stimulates hypothalamic GnRH release *in vitro* (human fetus: Rasmussen *et al.*, 1983; rats: Wilkes and Yen, 1981; Leadem *et al.*, 1985; Nikolarakis *et al.*, 1986; Kalra *et al.*, 1987; Rasmussen *et al.*, 1988) and *in vivo* (rabbits: Orstead and Spies, 1987; rat: Ching, 1983; Sarkar and Yen, 1985) and blocks the opiate suppression of GnRH release *in vivo* (rats: Ching, 1983). Ferin *et al* (1984) proposed that fluctuation in hypothalamic β-END activity is essential for menstrual cyclicity and that amenorrhea may arise when this cyclic change in β–END is disturbed. Current evidence indicates that EOPs modulate GnRH secretion, at least in part, by presynaptic contact on GnRH terminals in the ME (Rostene *et al.*, 1982) as well as by presynaptic inhibition of hypothalamic adrenergic systems in the rat (Leadem *et al.*, 1985; Kalra *et al.*, 1987). EOP neurons may also mediate the inhibitory actions of gonadal steroids on GnRH release in rats (Nikolarakis *et al.*, 1986b, Karahalios and Levine, 1988) and in birds (cited in Stansfield and Cunningham, 1988). The presence of ACTH (17-39)-like (guinea pig: Tramu *et al.*, 1977; Beauvillain *et al.*, 1981) and β–END-like (human: Leonardelli and Tramu, 1979) immunoactivity has been reported in the preoptic region; the physiological significance is not known.

Gamma-Aminobutyric acid. Evidence suggests that GABAergic interneurons mediate the negative feedback effects of gonadal steroids on LH secretion by tonically inhibiting the activity of the GnRH pulse generator (Honma and Wuttke, 1980; Fuchs *et*

al., 1984; Demling *et al.*, 1985; Flugge *et al*, 1986). GABA containing terminals have been observed to synapse with GnRH neurons in preoptic region of rats (Leranth *et al.*, 1985a, 1988).

Vasoactive intestinal peptide. Central vasoactive intestinal peptide (VIP) has also been implicated in the regulation of GnRH neuronal activity. Studies show that VIP can stimulate GnRH release from a hypothalamic synaptosomal preparation (Samson *et al.*, 1981) and from the MBH-pituitary in a double chamber perifusion system (Ohtsuka *et al.*, 1988). However, intraventricular injection of purified VIP (Vijayan *et al.*, 1979), but not intraventricular infusion of synthetic VIP (Alexander *et al.*, 1985) increases circulating levels of LH in OVX rats.

Corticotropin-releasing factor. Recent studies have suggested that corticotropin-releasing factor (CRF) mediates the stress-induced inhibitory effects on reproductive functions through suppression of GnRH secretion (Rivier and Vale, 1984; Rivier *et al.*, 1986). This view is supported by the ability of CRF to inhibit both basal and depolarization-induced GnRH release from the MBH *in vitro* (Gambacciani *et al* , 1986; Nikolarakis *et al.*, 1986a). Also, a CRF receptor antagonist increases GnRH release *in vitro* and *in vivo* in male rats (Nikolarakis *et al.*, 1988). Direct synaptic contacts between CRF- and GnRH- containing neurons in the preoptic region of the rat have been demonstrated (MacLusky *et al*, 1988). Other evidence suggests that CRF may inhibit GnRH release by stimulating the release of opioid peptides from the MBH (Nikolarakis *et al.*, 1988).

In summary, GnRH neuronal activities are affected by different neurotransmitters and neuropeptides through actions at the level of the cell bodies and at the level of the nerve terminals in the ME (see Negro-Vilar, 1982; Jacobowitz, 1988; Silverman, 1988). The magnitude or the direction of the GnRH response to these neural inputs, however, is influenced by the gonadal steroid milieu (see Kalra and Kalra, 1983). Studies in mammals

clearly demonstrate that the hypothalamic GnRH neurons constitute a common pathway through which neural inputs and ovarian signals interact to regulate pituitary LH secretion (see Fink, 1988).

1.2 Brain GnRH neuronal system and its regulation in teleosts

1.2.1 Characterization of GnRH peptides

In teleosts, gonadotropin-releasing hormone (GnRH) activity has been demonstrated in crude hypothalamic extracts *in vitro* (Breton *et al.*, 1972) and *in vivo* (Breton *et al.*, 1975; Crim *et al.*, 1976). However, it was not until 1983 that one of the major molecular forms of GnRH in salmon brain was isolated and characterized as pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2 (Sherwood *et al.*, 1983), a decapeptide with two amino acids different from that of the mammalian GnRH. Subsequent studies showed that sGnRH is found as a major form in a wide range of species (see Millar and King, 1987; Sherwood and Lovejoy, 1989), including goldfish (Sherwood and Harvey, 1986), but is absent in the African catfish (Sherwood *et al.*, 1989). Although multiple forms of GnRH have been shown in goldfish, as in other teleosts, the different GnRH forms have not been well characterized.

1.2.2 Immunocytochemical localization of GnRH in the teleost brain

Immunocytochemical studies in the goldfish using an antiserum raised against synthetic sGnRH showed that ir-GnRH cell bodies were distributed starting anteriorly in the olfactory nerves and bulbs, continuing along the ventral telencephalon, through the ventrolateral preoptic region and into the anteroventral hypothalamus; ir-GnRH fibers were found throughout the brain areas described above in which GnRH cell bodies were located, as well as in other brain areas (Kah *et al.*, 1986). GnRH cell bodies were also found in the anterior part of the dorsomedial midbrain tegmentum (Kah *et al.*, 1986). Studies in

other teleosts also localized ir-GnRH cell bodies in the anteroventral preoptic region although the distribution of GnRH immunoreactivity in other brain areas is less uniform among fishes (see Peter, 1986).

The ir-GnRH cell bodies and fibers in the olfactory bulbs and the medial olfactory tracts (mOT) of goldfish (Kah *et al.*, 1986) belong to a part of the terminal nerve system. Because of the central projections of TN in medial nuclei of the ventral telencephalon (supracommissural nucleus of area ventralis telencephali, Vs, and ventral nucleus of area ventralis telencephali, Vv; Demski and Northcutt, 1983; Levine and Dethier, 1985; von Bartheld and Meyer, 1986) which are known to be important for spawning behavior in male goldfish (Kyle and Peter, 1982), TN in the mOT has been proposed to function as a chemosensory link between various environmental cues and reproductive behavior (Demski and Northcutt, 1983; Stell and Walker, 1987). The involvement of mOT in mediating behavioral and hormonal responses to pheromonal stimuli has been suggested in teleosts (African catfish: Resnik, 1988; goldfish: Stacey and Kyle, 1983). However, it is not known whether the olfactory nerve or the TN in the mOT mediates the input of pheromones; the terminal fields of the secondary olfactory fibers in mOT overlap those of the TN in the ventral telencephalon and the anterior preoptic areas in goldfish (von Bartheld *et al,* 1984; Levine and Dethier, 1985).

In platyfish, the nucleus olfactoretinalis (NOR), a part of the TN system, is the first region in the brain in which ir-GnRH can be detected during ontogeny; ir-GnRH appears later in the nucleus preopticus paraventricularis (NPP) and nucleus lateralis tuberis (NLT) when gonadal development has already initiated (Halpern-Sebold and Schreibman, 1983). It was suggested that NOR may mediate the environmental inputs for the maturation of the GnRH neuronal system during puberty (Halpern-Sebold and Schreibman, 1983).

Unlike other tetrapods in which the pars distalis of the pituitary is connected with the neurophypophysis through the hypothalamo-hypophyseal portal vascular system, neurosecretory fibers directly innervate the pars distalis of the pituitary in teleosts (see

Holmes and Ball, 1974; Batten and Ingleton, 1987). Generally, neurohormones are released from nerve terminals located near or in direct synaptoid contact with the pituitary cells, or from terminals separated from the pituitary cells by a basement membrane (see Batten and Ingleton, 1987; Peter *et al.*, 1989). In goldfish, gonadotrophs in the pars distalis of the pituitary are directly innervated by aminergic and peptidergic fibers; however, peptidergic fibers (type A fibers according to Knowles and Vollrath, 1966) seldom make direct synaptoid contact with the gonadotrophs (Leatherland, 1972; Kaul and Vollrath, 1974). A direct innervation of gonadotrophs by ir-GnRH fibers has been demonstrated in catfish (Peute *et al.*, 1987) and molly (Batten, 1986). The ir-GnRH fibers in the pars distalis of the goldfish pituitary have been shown to originate primarily from cell bodies in the preoptic region (Kah *et al.*, 1986).

1.2.3 Regulation of GnRH neuronal activity during spawning

Changes in plasma GtH and steroid hormone levels during the spawning period have been described in teleosts, including several salmonid species (see Fostier *et al.*, 1983; Scott, 1987), white sucker (Scott *et al*, 1984; Stacey *et al.*, 1984), carp (Santos *et al.*, 1986), and goldfish (Kobayashi *et al.*, 1986, 1987). Goldfish, like other annual and multiple spawning teleosts, have a surge increase in GtH secretion (Stacey *et al.*, 1979a; Kobayashi *et al*, 1988) that mediates the processes of final oocyte maturation and ovulation (see Nagahama, 1987). In salmonids, such as rainbow trout, a gradual and prolonged rise in plasma GtH levels occurs over and after the entire periovulatory period (Jalabert and Breton, 1980).

Studies on the changes in brain GnRH levels associated with the stimulation of pituitary GtH secretion during spawning in teleosts are limited to brown trout (Breton *et al.*, 1986) and roach (Breton *et al.*, 1988a,b). In male and female roach, an inverse relationship between brain GnRH and serum GtH levels has been observed during the

spawning period (Breton *et al.*, 1988a,b). However, a significant change in the total brain GnRH content was not found in brown trout during the periovulatory period (Breton *et al.*, 1986).

1.2.4 Behavioral regulation of GnRH neuronal activity in teleosts

Behavioral or pheromonal activation of pituitary GtH secretion has been demonstrated in male goldfish (Kyle *et al.*, 1985). Male goldfish will behaviorally interact with females which are induced to perform spawning behavior by intramuscular injection of prostaglandin (PG) $F_{2\alpha}$, and have small, but significant increases in serum GtH levels during this behaviorial interaction (Kyle *et al.*, 1985). However, exposure of male goldfish to spontaneously ovulating females induces a marked increase or surge in serum GtH levels (Kobayashi *et al.*, 1986; Stacey *et al.*, 1989). In a series of studies, Stacey and coworkers demonstrated that the increase in serum GtH levels during spawning in male goldfish could be accounted for by two pheromones released in temporal sequence from ovulating females: a preovulatory primer pheromone, 17α20β-dihydroxy-4-pregnen-3-one (17,20β-P) and a postovulatory releaser pheromone, prostaglandin (Stacey and Sorensen, 1986; Dulka *et al.*, 1987; Sorensen *et al.*, 1988; Stacey *et al.*, 1989). It was also shown that exposure of male goldfish to 17,20β-P alone rapidly increases circulating GtH levels, whereas exposure to PG or PG-treated females (which presumably release PG pheromones) induces an increase in male serum GtH only after behavioral interaction with PG-treated fish (Sorensen *et al.*, 1989). Behavioral or pheromonal stimulation of the pituitary-gonadal axis have been suggested in other teleosts (see Colombo *et al.*, 1982; Liley and Stacey, 1983; Lambert *et al.*, 1986; Demski, 1987; Stacey , 1987; Stacey *et al.*, 1987). However, little is known about the possible role of GnRH in the behavioral and/or pheromonal stimulation of reproductive activities in teleosts (see Dulka, 1989).

1.2.5 Gonadal regulation of GnRH neuronal activity in teleosts

In salmonid species, plasma estradiol-17β (E_2) levels decrease prior to and during the periovulatory period (Fostier *et al.*, 1978; Scott *et al.*, 1983; Yamauchi *et al.*, 1984). E_2 reduced plasma GtH levels, whereas ovariectomy increased GtH secretion in trout (Bommelaer *et al.*, 1981). These results have led to the suggestion that reduced negative feedback effects of E_2 induce the GtH rise and ovulation in salmonid species (see Fostier *et al.*, 1978; Scott *et al.*, 1983; Goos, 1987). Negative feedback effects of gonadal steroids on GtH release have been demonstrated using gonadectomized African catfish (de Leeuw *et al.*, 1986) and rainbow trout (Billard *et al.*, 1977; van Putten *et al.*, 1981).

In contrast, circulating E_2 levels increase at the time of the ovulatory surge in goldfish (Kobayashi *et al.*, 1987) and in white suckers during spawning (Scott *et al.*, 1984). Moreover, studies showed that goldfish have a GtH surge and ovulate even when E_2 levels are artificially increased (Pankhurst and Stacey; 1985; Kobayashi *et al.*, 1987). In female goldfish, a gradual decrease in estradiol and an increase in T occurs towards the end of vitellogenesis (Kagawa *et al.*, 1983; Kobayashi *et al.*, 1986). Kobayashi *et al.*, (1989a,b) showed that an increase in temperature induces a preovulatory surge-like increase in GtH levels in T and E_2 implanted OVX or sexually regressed female goldfish, but not in OVX fish. This finding, together with the observation that high T levels were found in the goldfish undergoing ovulation, suggest that elevated levels of T are important for the induction of the preovulatory GtH surge. Environmental factors, such as water temperature, and the presence of spawning substrate, have been shown to be important for the preovulatory surge of GtH to occur in female goldfish (Stacey, *et al.*, 1979a), whereas photoperiod influences the timing of the surge (Stacey *et al*, 1979b). The positive feedback effect of T is presumably exerted at the brain and/or pituitary level to allow the environmental (temperature and photoperiod) cues to ultimately trigger the preovulatory GtH surge (see Aida, 1988).

The sites of gonadal steroid feedback on the brain-pituitary axis to influence GtH

secretion in teleosts are unknown. Evidence for gonadal steroid action on GnRH neurons in teleosts comes primarily from studies on immature fish in which positive steroid feedback on GtH secretion is predominant (see Goos, 1987). Treatment with human chorionic gonadotropin (hCG) increased brain and pituitary GnRH contents in male eels (Dufour *et al.*, 1985); immunocytochemical studies showed a large increase in the number and density of ir-GnRH terminals in the neurohypophysis of the hCG-treated eels (Kah *et al.*, 1988). Treatment with carp pituitary extracts also increased the pituitary GnRH content of female silver eels, and the effects were abolished by ovariectomy (Dufour *et al.*, 1989). Moreover, chronic treatment with estradiol increased the pituitary GnRH content in female silver eels (Dufour *et al.*, 1985) while testosterone implants increased the GnRH bioactivity in the telencephalon and diencephalon extracts of immature rainbow trout (Goos *et al.*, 1986). All these studies suggest that positive feedback of E_2 on GtH secretion in immature eels (Olivereau and Olivereau, 1979a,b; Dufour *et al.*, 1983; Counis *et al.*, 1987) and in juvenile rainbow trout (Crim and Evans, 1979; Trinh *et al.*, 1986) may be mediated through brain GnRH neurons, as well as directly on the pituitary (Fahraeus-van Ree *et al.*, 1983). Nevertheless, steroid binding sites in the ventral telencephalon and preoptic region of goldfish (Kim *et al.*, 1978) have a distribution in proximity to, but not overlapping that of the GnRH neurons (Kah *et al.*, 1986), suggesting that the effects of sex steroids on the GnRH neuronal system may be mediated by other neurotransmitters or neuropeptides as has been shown in mammals (see Fink, 1988). Regulation of brain levels of DA by gonadal steroids has been described in toadfish (Pennipacker *et al.*, 1985).

1.2.6 Involvement of neurotransmitters and neuropeptides in regulation of GtH secretion in teleosts

An inhibitory role of DA on basal and GnRH-induced pituitary GtH secretion have also been shown in goldfish and in other teleosts (see Peter *et al.*, 1986; de Leeuw *et al.*,

1987). In goldfish, a dopaminergic preoptic-hypophyseal pathway has been identified and is thought to be the neural substrate for the dopaminergic inhibition of GtH secretion at the level of pituitary (Kah *et al.*, 1987). A direct inhibitory action of DA or DA receptor agonists on pituitary GtH secretion in goldfish has been confirmed in studies using cultured pituitary cells (Chang *et al.*, 1984, 1989; Chang *et al.*, submitted). However, in the African catfish, the DA receptor agonist, apomorphine, inhibits *in vitro* LHRH analog-induced GtH release, but not basal GtH secretion from cultured pituitary fragments in perifusion (de Leeuw *et al.*, 1986), suggesting that DA may interfere with the GnRH receptor or post-receptor mechanism on pituitary GtH secretion.

The influence of NE, 5-HT, and NPY on pituitary GtH secretion has also been studied in goldfish. It was found that pituitary GtH secretion is stimulated by NE (Chang *et al.*, 1984; Chang and Peter, 1984), 5-HT (Somoza *et al.*, 1988) and NPY (Peng *et al.*, submitted) in goldfish.

Recent immunohistochemical studies in teleosts have confirmed the presence of many neuronal fibers containing tyrosine hydroxylase (Yoshida *et al.*, 1985; Hornby *et al.*, 1987), DA (Kah *et al.*, 1984, 1987; Meek *et al.*, 1989), NE (Ekström *et al.*, 1986), and 5-HT (Kah and Chambolle, 1983; Frankenhuis-van de Heuvel and Nieuwenhuys, 1984; Margolis-Kazan *et al.*, 1985; Meek and Joosten, 1989) in the preoptic region and the pars distalis of the pituitary. However, there is no published information concerning the possible influence of monoamines on the GnRH neuronal system in this vertebrate group.

1.3 Purpose of this study

The major objective of the present study is to investigate the regulation of the brain GnRH activity in goldfish under different physiological conditions. This thesis consists of seven independent studies, each of which constitutes a full Chapter on its own; the last

Chapter presents a General Discussion of the results from all the studies.

Chapter 2 describes the development and validation of a GnRH RIA using an antiserum raised against synthetic sGnRH. Using this GnRH RIA, the temporal sequence of changes in brain and pituitary GnRH levels which precede and accompany the preovulatory surge of GtH are described. Alterations in brain and pituitary GnRH levels in male goldfish during spawning behavior with PG-treated females were also studied (Chapter 3). In Chapter 4, results from *in vivo* experiments indicate that brain and pituitary levels of GnRH are regulated by a central dopaminergic system. Using male goldfish during spawning behavior with PG-treated females as the physiological model, the inhibitory influences of the dopaminergic system on brain GnRH neurons and pituitary GtH secretion were also demonstrated. In Chapter 5, the temporal changes in brain and pituitary GnRH levels in males during spawning with spontaneously ovulating females were described, and compared to the changes in females during the periovulatory period. Chapter 6 describes the development and validation of an *in vitro* static incubation system for studying the direct effects of DA, NE and 5-HT on the GnRH release from slices of the preoptic-anterior hypothalamus and fragments of the pituitary in goldfish. The involvement of specific adrenergic and dopaminergic receptor subtypes on GnRH release from preoptic-anterior hypothalamic slices and pituitary fragments *in vitro* were examined in Chapter 7. Chapter 8 describes the biochemical characterization of the second molecular form of GnRH in goldfish brain and demonstrates that the two forms of GnRH have a differential distribution in the brain and pituitary. In Chapter 9, the major findings of the studies in this thesis are summarized and discussed to provide a model in goldfish in which the interaction between the GnRH neuronal system and other neurotransmitters can be studied in relation to pituitary GtH secretion under different physiological conditions.

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2. BRAIN DISTRIBUTION OF RADIOIMMUNOASSAYABLE GONADOTROPIN RELEASING HORMONE IN FEMALE GOLDFISH: SEASONAL VARIATION AND PERIOVULATORY CHANGES [1]

2.1 INTRODUCTION

Recently, a gonadotropin-releasing hormone (GnRH) in chum salmon brain has been determined to be [Trp^7, Leu^8]-GnRH (sGnRH, Sherwood *et al.*, 1983); the basic structure of GnRH is defined as pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. This decapeptide is found in a wide range of teleost species (Breton *et al.*, 1984; 1986; King and Millar, 1985; Powell *et al.*, 1986; Sherwood *et al.*, 1984), including goldfish (Breton *et al.*, 1986; Sherwood and Harvey, 1986). Other molecular forms of immunoreactive GnRH have been reported in brain extracts of teleosts by high pressure liquid chromatography (HPLC) and radioimmunoassay (RIA) analysis, specifically [Gln^8]-GnRH (cGnRH-I) in *Tilapia sparrmanii* (King and Millar, 1985) and [His^5, Trp^7, Tyr^8]-GnRH (cGnRH-II) in *Coris julis* (Powell *et al.*, 1986). In goldfish brain, the presence of sGnRH and a second ir-GnRH form have been demonstrated by chromatographic and immunological criteria (Sherwood and Harvey, 1986). The second ir-GnRH form in goldfish brain extract has the same elution pattern as cGnRH-II in a HPLC system, and, by the same criterion, the mammalian form of GnRH (mGnRH), cGnRH-I and [Tyr^3, Leu^5,

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Glu^6, Trp^7, Lys^8]-GnRH (lamprey GnRH) are not present in brain extracts of goldfish (in Chapter 8; Sherwood and Harvey, 1986).

The immunocytochemical distribution of sGnRH neurons in the brain of goldfish has been described (Kah *et al.*, 1986), using in part the same sGnRH antiserum as in the present study. Immunoreactive GnRH cell bodies were found in a continuum ranging from the olfactory nerve and bulbs, through the medial olfactory tract, the ventromedial telencephalon, the ventrolateral preoptic region to the ventrolateral anterior hypothalamus. GnRH cell bodies were also found in the rostral midbrain tegmentum. Immunoreactive GnRH fibers were found throughout the brain areas in which GnRH cell bodies were located, as well as in a wide range of other brain structures. A similar organization of GnRH structures has been found in other vertebrates (for review see Peter, 1986).

Using a radioimmunoassay system for mGnRH, the GnRH content in the pituitary and brain of immature female European eels (Dufour *et al.*, 1982) and sexually mature female platyfish have been quantified (Schreibman *et al.*, 1983). In brown trout, the GnRH content of the brain and the pituitary was determined during the periovulatory period using a sGnRH radioimmunoassay (Breton *et al.*, 1986). The study showed that there was an increase in the pituitary GnRH content in ovulating or freshly ovulated brown trout compared to fish in the final stages of gonadal maturation. In addition, the study showed an increase in the total brain GnRH content from the end of vitellogenesis to the final stage of gonadal maturation, although it failed to detect a significant change in the total brain GnRH content during the periovulatory period.

In the present study, the total immunoreactive GnRH content in discrete brain areas of female goldfish was determined by a radioimmunoassay using a rabbit anti-sGnRH antiserum as primary antibody and sGnRH as standards. The purpose of this study was to validate the radioimmunoassay for determination of GnRH in discrete brain areas of goldfish and to determine whether there are seasonal variations and temporal changes

during spontaneous ovulation in GnRH concentrations in discrete brain areas in the female goldfish.

2.2 MATERIALS AND METHODS

Experimental animals.

Goldfish, 7 - 10 cm standard length, were purchased from Grassyfork Fisheries Company, Martinsville, Indiana at various times of the year. The fish were held in an 1800-liter flow-through aquarium at 12-13°C on a simulated natural (Edmonton) photoperiod for one to two weeks. In Experiment I, seasonal variations of brain GnRH content were studied; sexually regressed (September), recrudescing (January) and sexually mature/prespawning (April) females were selected and acclimated in 96-liter flow-through aquaria under simulated natural photoperiods for 10 days at either 10°C or 18°C. In Experiments II (May, 1985) and III (May, 1986), the changes of brain GnRH content during spontaneous ovulation were studied. Females with a distended and soft abdomen were selected and held at 12°C. Spontaneous ovulation was induced by conditions similar to those described previously (Stacey *et al.*, 1979). Briefly, the procedure involved an increase in water temperature from 12°C to 21°C over a period of 10 hours (from 1600 hr Day 1 to 0200 hr Day 2), the addition into each aquarium of artificial floating vegetation (1600 hr Day 1), and one or two spermiating male goldfish along with a female treated with prostaglandin F2α (at 0800 hr Day 2) so that there was a spawning behavior stimulus for the preovulatory females. In the control aquaria, temperature was increased similarly, but no artificial floating vegetation and no sexually active male and female goldfish were introduced.

For blood and brain sampling, fish were anesthetized in 0.05% tricaine methanesulfonate. Blood was sampled from the caudal vasculature and collected serum

was frozen on dry ice and stored at -30°C. Serum samples were analyzed by a radioimmunoassay for GtH as described previously (Peter *et al.*, 1984). The brain was rapidly removed after blood sampling, and different brain parts (olfactory bulbs, telencephalon, hypothalamus, optic tectum-thalamus, cerebellum, medulla; see Fig. 2.1), a 0.5 cm segment of anterior spinal cord and the pituitary were rapidly dissected on a glass petri dish over ice, and each fragment immersed in 2 ml of ice-cold 2 M acetic acid.

In Experiment I (seasonal study) fish were sampled at between two to five hours after lights on. In Experiments II and III (ovulation study), fish were sampled from the control and ovulation aquaria at various times from 1300 hour on Day 1 to 1300 hour on Day 4 as indicated in Figs. 2.4, 2.5, 2.6, and 2.7. At 1300 hour on Day 2, fish sampled from the ovulation aquaria were divided into two groups based on their serum GtH levels; individual fish in which serum GtH levels were significantly higher (Student's t-test for single samples) than the mean GtH levels of the control fish were considered to be in the initial stage of the ovulatory surge of GtH and were designated as "ovulatory" fish in which serum GtH levels did not differ significantly from the control fish were designated as "nonovulatory". At 0600 hr and 0800 hr on Day 3, ovulation was confirmed by lightly squeezing the abdomen of fish to eject a stream of oocytes from the ovipore.

Extraction of brain GnRH.

Different brain parts were homogenized separately in 2 ml ice-cold 2 M acetic acid by use of a "Polytron" (in Experiment I) or by sonication (in Experiments II and III). The homogenate was centrifuged at 10,000 g for 45 min. at 4°C, and the supernatant frozen and lyophilized before storage at -30°C. For the brain samples in Experiment II and III, an aliquot (100 µl) of the homogenate was removed for protein determination according to Bradford's method (BioRad) prior to centrifugation. In Experiment I, protein content of the brain parts was not determined, and the following equation was used to normalize the GnRH content per brain fragment to a 25 g gonad free-body weight fish:

GnRH content

= [(total GnRH measured in the brain fragment x 25) / body weight] $^{0.5}$

From the data in other experiments, it was found that there was a linear relationship between the protein content of the brain parts and the square root of the gonad free-body weight (data not shown).

Before radioimmunoassay, lyophilized brain extracts were reconstituted in the assay buffer and centrifuged at 14,000g for 30 min at 4°C. After centrifugation, the supernatant was used for RIA. It was found that a second extraction of the homogenate pellet after centrifugation with 2 ml 2 M acetic acid yielded less than 5 % of the immunoactivity found in the first extraction, indicating that a single extraction was adequate. To estimate the recovery from extraction, sGnRH or ^{125}I-sGnRH was added to triplicate aliquots of brain homogenate prior to the first centrifugation and the lyophilization steps. As more than 87% (87.6 - 94.2%) of the sGnRH or radioactivity (^{125}I-sGnRH) was recovered in the supernatant of the reconstituted extracts of various brain parts, no correction factor was applied to extraction efficiency.

Radioimmunoassay of GnRH.

The salmon GnRH was synthesized by J. Rivier and W. Vale, and its bioactivity was tested in goldfish both *in vitro* (MacKenzie *et al.*, 1984) and *in vivo* (Peter *et al.*, 1985). sGnRH was iodinated using the Chloramine T method. One mCi Na ^{125}I in 50 µl of 0.5M phosphate buffer (pH 7.5), and 10 µg Chloramine T (Sigma) in 10 µl of 0.05 M phosphate buffer were added to 5 µg of sGnRH in 10 µl of 0.1 M acetic acid, and incubated at room temperature for 1 min with constant agitation. The reaction was stopped by the addition of 200 µl starting elution buffer (0.002 M ammonium acetate buffer, pH = 4.5). A CM-Biogel A (BioRad) cation exchange column (1.1 x 8 cm) was used to purify the labelled sGnRH; the column was eluted with 10 ml 0.002 M ammonium acetate buffer

(pH = 4.5) and then 30 ml of 0.06 M ammonium acetate buffer (pH = 4.5) in one-step change. Using this column, the radioactive free iodide was eluted first, followed by the labelled sGnRH peak. The specific activity of the ^{125}I-sGnRH was estimated by radioimmunoassay to be about 860 μCi/μg.

GnRH in extracts of brain fragments and other tissues was measured by double-antibody radioimmunoassay using a rabbit antiserum (PBL-49) raised against sGnRH coupled to human alpha-globulins with bisdiazotized benzidine at The Clayton Foundation Laboratories, according to the method of Vale *et al* (1983). Assay buffer consisted of 0.08 M barbital buffer, pH=8.6, 0.01% merthiolate (Sigma) and 0.5% bovine serum albumin (Sigma). A 100 μl aliquot of sample or standard sGnRH was added to 200 μl of rabbit anti-sGnRH serum containing 2.5 % normal rabbit serum and 200 μl of ^{125}I-sGnRH (approximately 15,000 cpm/tube); the final dilution of the anti-sGnRH serum was 1:250,000. Incubation was for 48 hours with periodic agitation. Precipitation of the antibody-bound hormone was done by addition of 200 μl of a 1:20 initial dilution of goat anti-rabbit gamma-globulin (Arnel Products) and incubation overnight at 4°C. The tubes were then centrifuged for 25 min at 1,000g, the supernatant decanted, and the radioactivity in the bound fraction determined. Specific binding of ^{125}I-sGnRH ranged from 20 to 40%. Within assay coefficient of variation was 9.8 % (n=5) for samples at 10 pg/tube and 11.2% (n=5) for samples at 80 pg/tube. Interassay variation ranged from 12.2% (n=5) for 10 pg/tube to 17.5% (n=5) for 80 pg/tube. The dose at which relative percentage binding (B/Bo) equals 50% is approximately 40 pg. The minimum detectable level of the assay, defined as the mean plus two standard deviations for the reading of zero blank tubes was 0.40 ± 0.16 pg/tube (n=17).

To determine the nonspecific effect of brain extracts on the RIA, known amounts of sGnRH were added to aliquots of a reconstituted brain extract pool. Recovery of added synthetic sGnRH in the RIA was not significantly different from 100 %. Furthermore,

addition of bacitracin (0.1% w/v, Sigma), a GnRH degradative peptidase inhibitor (McKelvy *et al.*, 1976), had no effect on the assayed values of sGnRH (from about 20 to 300 pg per tube) in the brain extracts or on the slopes of the standard curves. To determine whether there was immunoactivity in the macromolecular component of acid extracts of the brain and pituitary, the extracts of different brain parts collected in September and synthetic sGnRH were separately chromatographed on a Sephadex G-25 column (1.1 x 20 cm), and each fraction (0.5 ml) was lyophilized and reconstituted in the assay buffer and assayed for sGnRH by RIA. Briefly, the GnRH immunoactivity in different brain areas coeluted with the synthetic sGnRH in this chromatographic system. No immunoactivity was detected in the macromolecular component of the extracts eluted in the void volume, indicating that the antiserum does not crossreact with the larger molecular precursor of GnRH, demonstrated to be present in mammals (Seeburg and Adelman, 1984).

Acid extracts of various brain parts and pituitaries produced displacement curves which were parallel to those of the synthetic sGnRH in the RIA (Fig. 2.2). Goldfish serum (100 µl per tube) and acid extracts of serum (100 µl per tube), muscle, kidney, hepato-pancreas, testis and ovary (40 mg fresh weight per tube) had no significant crossreactivity in the RIA.

The antiserum was found to crossreact with both forms of GnRH described previously (Sherwood and Harvey, 1986) although sGnRH constitutes more than 70% of the crossreactive material detected in the RIA (Chapter 8). The antiserum also crossreacts with other known forms of GnRH in vertebrates, specifically mGnRH (Sigma), cGnRH-I (synthesized by R. Millar) and to a lesser extent with cGnRH-II and lamprey GnRH (both synthesized by R. Millar). The displacement curves produced by cGnRH-I, cGnRH-II and lamprey GnRH were not parallel to the sGnRH standard; the lamprey GnRH gave only incomplete label displacement (Fig. 2.2). There was no significant crossreactivity with the following peptides: melanocyte-stimulating hormone (MSH), somatostatin (SRIF), met-

enkephalin, FMRFamide, thyrotrophin-releasing hormone (TRH), β-endorphin, vasoactive intestinal peptide (VIP), neurotensin and substance P (all from Sigma); urotensin I and II (provided by K. Lederis); free acid form of sGnRH (GnRH-C), [D-Arg^6, Trp^7, Leu^8, Pro^9 Net]-GnRH, [D-Ala^6, Pro^9 Net]-GnRH (GnRH-A), $GnRH_{1-5}$, $GnRH_{1-7}$ and $GnRH_{5-10}$ (synthesized by J. Rivier and W. Vale). Crossreactivity of the various GnRH analogues to the antiserum was determined by comparing the dose at which 50% of the specific binding of ^{125}I-sGnRH in the zero hormone tube was inhibited.

Statistical analysis.

The data were analysed using the Student's t-test, or by one-way analysis of variance and Duncan's multiple range test after logarithmic transformation.

2.3 RESULTS

Seasonal Variations

The changes in GnRH content in discrete brain areas during the seasonal gonadal cycle in female goldfish are presented in Table 2.2. Significant seasonal variations were found in the hypothalamus, pituitary and spinal cord. In fish acclimated to 10°C, the GnRH content of the hypothalamus was lower in sexually mature fish compared to sexually regressed fish, but not significantly different from that of sexually recrudescing fish. In fish acclimated to 18°C, the pituitary GnRH was lower, but the spinal cord GnRH content was higher, in sexually mature fish compared with those in sexually regressed fish. There were no significant variations in the GnRH content in other regions of the brain. Also, there were no significant differences in the GnRH content in different brain areas of sexually regressed fish acclimated to 10°C and 18°C, and of sexually mature fish acclimated to 10°C and 18°C, except that the GnRH content in the hypothalamus of sexually mature fish was higher at 18°C than at 10°C.

Periovulatory Changes

Experiment II The serum GtH changes during spontaneous ovulation are shown in Fig. 2.4. At 1300 hr on Day 2, control fish had serum GtH levels similar to that at 1300 on Day 1. In the ovulation group, 6 fish had significantly higher serum GtH levels compared to the control fish at 1300 hr on Day 2 and were designated as ovulatory fish; 10 fish had serum GtH levels similar to those of the control fish were designated as nonovulatory. Fish that were ovulated at 0600 hr on Day 3 were found to have high serum GtH levels, indicative of the ovulatory surge of GtH. At 1300 hr on Day 3, the average serum GtH level of the ovulated fish was higher than the average level found in the controls. Serum GtH levels in ovulated fish were similar to the levels found in normal control fish at 1300 on Day 4.

The preovulatory changes of GnRH concentration in discrete brain areas of goldfish at 1300 hr on Day 2 are shown in Fig. 2.3. At this sample time, there was a significant decrease in the GnRH concentration in the olfactory bulbs and the telencephalon of the ovulatory fish compared to the control fish. When comparison was made between the ovulatory and nonovulatory fish at 1300 hr on Day 2, the ovulatory fish were found to have a significant decrease in the GnRH concentration in the olfactory bulbs and telencephalon as well as the hypothalamus, optic tectum-thalamus, and pituitary (Figs. 2.3; 2.4, 2.5). There were no significant differences between the control and nonovulatory fish in the GnRH concentrations found in the other various brain areas, except for a significant decrease in the olfactory bulbs of the nonovulatory fish. The GnRH concentrations in the cerebellum, medulla and spinal cord showed no significant variations among the control, ovulatory and nonovulatory fish.

Temporal changes in the GnRH concentrations in olfactory bulbs, telencephalon, hypothalamus and pituitary during the periovulatory period of goldfish are shown in Figs. 2.4, 2.5. At 0600 hr on Day 3, when the serum GtH levels were highest, the GnRH

concentrations in the olfactory bulbs, telencephalon and pituitary of the ovulated fish were significantly lower than in the control fish.

At 1300 hr on Day 3, there were no significant differences between the ovulated and the control fish in the GnRH concentrations in the various brain areas sampled. However, the GnRH concentrations in the pituitary of the ovulated fish at 1300 hr on Day 3 showed a significant increase compared to the levels found in the ovulated fish at 0600 hr on Day 3 (see Fig. 2.4).

At 1300 hr on Day 4 when the serum GtH levels in the ovulated and the normal control fish were similar, there were no significant differences in the GnRH concentrations of the various brain areas, pituitary or spinal cord between the two groups.

Experiment III. Changes of serum GtH levels and GnRH concentrations in discrete brain areas during the periovulatory period were similar to those observed in Experiment II (see Figs. 2.6, 2.7). However, no significant changes in GnRH concentrations in the optic tectum-thalamus were found in ovulatory fish at 1300 hr on Day 2 compared to the control or nonovulatory fish. Furthermore, there was no significant difference in the GnRH concentrations of the olfactory bulbs between the control and the nonovulatory fish (Fig. 2.6). On the other hand, a significant increase of the pituitary GnRH concentration in the nonovulatory fish was found compared to the control (Fig. 2.7).

2.4. DISCUSSION

Regional distribution of GnRH

The present study confirms the wide distribution of GnRH immunoactivity in the brain of female goldfish as shown by immunocytochemistry (Kah *et al.*, 1984, 1986). In teleosts, the equivalent of the median eminence is incorporated into the rostral

neurohypophysis of the pituitary (Holmes and Ball, 1974). Unlike the situation in mammals where over 90% of the total brain GnRH is found in the axon terminals in the median eminence (Selmanoff *et al.*, 1980), GnRH is more evenly distributed in the brain of goldfish and other teleosts. Such a wide distribution of GnRH in teleostean brain suggests multiple functions for GnRH other than as a gonadotropin releasing hormone in the pituitary.

In the present study, the pituitary of female goldfish normally contained only about 5 to 10% of the total brain GnRH, confirming the study by Sherwood and Harvey (1986) on smaller goldfish. Data from other teleost species, such as the adult mullet (Sherwood *et al.*, 1984), female brown trout (Breton *et al.*, 1986) and female platyfish (Schreibman *et al.*, 1983) also found that the pituitary normally contained not more than 15% of the total brain GnRH. However, in a study measuring the sGnRH immunoactivities in brain and pituitary of three teleost species, Breton *et al* (1984) reported average values of 5.51 ng GnRH/pituitary (n=2) and 10.95 ng GnRH/brain (n=2) in goldfish. The reason for this discrepancy is not understood although it may be related to the fact that the displacement curve by the serial dilution of goldfish brain extract was divided into two parts in Breton's RIA system with only the upper portion of the displacement curve parallel to that of the sGnRH standard. Using a heterologous mGnRH RIA system, Dufour *et al* (1982) found that the pituitary of the European eel contained about 25% of the total brain GnRH immunoactivity.

The presence of GnRH in the olfactory bulbs and the simultaneous changes of GnRH concentration in the olfactory bulbs with other brain areas during the periovulatory period (see later discussion) suggest that GnRH structures in the olfactory bulbs may serve important reproductive functions in this species. A possible role of GnRH in the olfactory bulbs in the regulation of maturation and differentiation of the pituitary-gonadal axis has been suggested for the platyfish (Halpern-Sebold and Schreibman, 1983), rat (Schwanzel-

Fukuda, 1985) and guinea pig (Schwanzel-Fukuda, 1981).

Seasonal variation

This study failed to show a clear parallel change of brain GnRH content with the seasonal ovarian development of goldfish. Instead, the GnRH contents in the hypothalamus and pituitary were higher in sexually regressed fish compared to sexually mature fish under certain temperature acclimation conditions. This suggests that regression of the ovary in goldfish is not associated with depletion of GnRH in the brain. In contrast, King and Millar (1980), using antiserum directed against mGnRH, detected GnRH immunoactivity in brain of gravid tilapia, but not in fish sampled during the non-breeding seasons. In a recent study, Gentile *et al* (1986) also reported that immunoreactive mGnRH content was higher in hypothalamic and telencephalic extracts from sexually mature females than in those from fish sampled in other non-breeding season. This discrepancy may be simply due to species difference or could be due to the fact that the antigenic requirements of the GnRH antiserum used in this study were different from those used in the other two studies.

Periovulatory Changes

In the ovulating fish, significant decreases in the GnRH concentrations found in the olfactory bulbs, telencephalon, hypothalamus, and pituitary (as early as 17 hr prior to ovulation) were correlated with increases in serum GtH levels. Such preovulatory decreases in brain GnRH concentrations, except for the olfactory bulbs in Experiment II, were not observed in fish that failed to ovulate. Notably, the GnRH concentrations in the olfactory bulbs, telencephalon, hypothalamus and pituitary returned to the normal control levels soon after ovulation. The brain areas showing periovulatory changes of GnRH concentration overlap with the apparent continuum of immunoreactive GnRH cell bodies and fibers from the olfactory bulb, along the ventral telencephalon, through the preoptic

region and the anterior hypothalamus to the pituitary. This may indicate that the GnRH neuronal system functions as an integrated unit for the activation of GtH secretion and subsequent induction of ovulation in goldfish.

A similar study of the periovulatory changes in brain GnRH levels is available only for brown trout (*Salmo trutta L*; Breton *et al.*, 1986); the pituitary GnRH content in trout in which germinal vesicle breakdown had occurred was lower than in fish in which the germinal vesicle was intact but at the periphery of the oocyte, and, in contrast, the pituitary GnRH content of the ovulating or recently ovulated fish was higher than the level found in fish at final stages of ovarian maturation. There were no significant changes in the total brain GnRH content during the periovulatory period, except that an increase occurred between the end of the vitellogenesis and the final stages of ovarian maturation. These findings suggest that in brown trout final maturation of oocytes is associated with an increase in brain GnRH and later biphasic changes in the pituitary GnRH content (a decrease followed by an increase).

Although both studies on female goldfish and brown trout demonstrated biphasic changes of pituitary GnRH during the periovulatory period, the timing and the direction of these changes relative to the periovulatory release of GtH were different. This may be related to the fact that the patterns of periovulatory release of GtH in these two species are different. In goldfish, serum GtH increases markedly at 8 to 12 hours prior to and reaches a peak level at about the time of spontaneous ovulation (Stacey *et al.*, 1979). The pituitary GnRH concentration decreased significantly at the time of ovulation when serum levels were at their peak, and returned back to the normal control levels soon after ovulation, concomitant with a sharp decrease in serum GtH levels. A similar relationship was not found in brown trout. Firstly, the preovulatory increase in serum GtH in salmonids, such as rainbow trout, is relatively small and serum GtH levels peak at about three weeks after ovulation (Jalabert *et al.*, 1980). In brown trout, a significant increase in serum GtH was

detected only in recently ovulated fish compared to the ovulating fish. Secondly, there was no significant change in pituitary GnRH content between ovulating and recently ovulated fish (Breton *et al.*, 1986).

Simultaneous changes in mGnRH content in discrete brain areas of the preoptico-suprachiasmatic-tuberoinfundibular (PTST) GnRH neuronal system associated with the ovulatory GtH surge have also been reported in rats (Wise *et al.*, 1981). In rats, there was an increase in the mGnRH concentrations in various brain nuclei in the PTST neuronal system before the ovulatory GtH surge occurred and a decrease in GnRH concentrations during the GtH surge (Wise *et al.*, 1981). Although the periovulatory changes of GnRH in discrete brain areas are different in goldfish and in rats, both studies suggest that the GnRH structures in different brain areas may function as an integrated unit.

The marked changes in GnRH concentration in the olfactory bulbs together with those in other brain areas during the periovulatory period provide the first evidence that the GnRH structures in the olfactory bulbs may be functionally related to the GnRH structures in other brain areas. One possibility is that the GnRH structures in the olfactory system serve as an important messenger to link environmental cues for ovulation to the central neuroendocrine system. Demski and Northcutt (1983) suggested that the GnRH-containing terminal nerve may be involved in triggering sexual responses to pheromones in goldfish. Changes in mGnRH concentrations in the olfactory bulbs in response to external chemical cues or pheromones had also been demonstrated in female voles (Dluzen *et al.*, 1981) and male mice (Dluzen and Ramirez, 1983).

This report represents the first study in a teleost to describe the temporal changes in GnRH concentrations in discrete brain areas and the pituitary in association with the serum GtH changes during the periovulatory period. The concomitant changes in GnRH concentrations in different brain areas and the pituitary suggests that various components of the GnRH neuronal system may function as an integrated unit, and are activated during

spontaneous ovulation in goldfish. Since this study failed to show clear seasonal changes in brain GnRH content parallel to seasonal ovarian recrudescence, the involvement of the central GnRH neuronal system in ovarian recrudescence requires further investigation. Nevertheless, it should be noted that the brain GnRH level reflects a balance between synthesis, release and degradation of the neuropeptide at any particular time and that further development of techniques to allow study of these processes will provide a better understanding of the function of the GnRH system.

TABLE 2.1

Relative activities of GnRH analogs and various peptides in GnRH radioimmunoassay system.

	1	2	3	4	5	6	7	8	9	10	Cross-reactivity [a] (%)
sGnRH	*p*Glu–	His–	Trp–	Ser–	Tyr–	Gly–	Trp–	Leu–	Pro–	Gly-NH_2	100
cGnRH-I	*p*Glu–	His–	Trp–	Ser–	Tyr–	Gly–	*Leu*–	*Gln*–	Pro–	Gly-NH_2	538 [b]
mGnRH	*p*Glu–	His–	Trp–	Ser–	Tyr–	Gly–	*Leu*–	*Arg*–	Pro–	Gly-NH_2	78
cGnRH-II	*p*Glu–	His–	Trp–	Ser–	*His*–	Gly–	Trp–	*Tyr*–	Pro–	Gly-NH_2	44 [b]
Lamprey GnRH	*p*Glu–	*His*–	*Tyr*–	Ser–	*Leu*–	*Glu*–	Trp–	*Lys*–	Pro–	Gly-NH_2	2 [b]
GnRH-C	*p*Glu–	His–	Trp–	Ser–	Tyr–	Gly–	Trp–	Leu–	Pro–	Gly-*OH*	<0.01
sGnRH-A	*p*Glu–	His–	Trp–	Ser–	Tyr–	*D-Arg*–	Trp–	Leu–	Pro-*Net*		<0.01
GnRH-A	*p*Glu–	His–	Trp–	Ser–	Tyr–	*D-Ala*–	*Leu*–	*Arg*–	Pro-*Net*		<0.01
$GnRH_{1-5}$	*p*Glu–	His–	Trp–	Ser–	Tyr-*OH*						<0.01
$GnRH_{1-7}$	*p*Glu–	His–	Trp–	Ser–	Tyr–	Gly–	*Leu-OH*				<0.01
$GnRH_{5-10}$				H–	Tyr–	Gly–	*Leu*–	*Arg*–	Pro–	Gly-NH_2	<0.01
α-MSH, SRIF, met-enkephalin, FMRFamide, TRH, β-endorphin, VIP, neurotensin, substance P, urotensin I and II [c]											<0.01

[a] Cross-reactivity was measured at B/B_0 = 50%; largest dose tested was 10 ng/tube.

[b] Nonparallel to sGnRH standard.

[c] Abbreviations defined in text.

TABLE 2.2

Seasonal Variations in GnRH Content in Discrete Brain Areas and Pituitary of Female Goldfish.

Month (gonadal condition)	Gonado-somatic index (%)	GnRH content * (pg)							
		Olfactory bulbs	Telen-cephalon	Hypo-thalamus	Optic tectum-thalamus	Cerebellum	Medulla	Spinal cord	Pituitary
				Acclimation temperature: 10°					
September (Regressed)	1.28 ± 0.42^{a}	107 ± 17	417 ± 103	397 ± 87^{b}	1310 ± 64	235 ± 41	361 ± 95	80 ± 36	90 ± 36
January (Recrudescing)	5.53 ± 0.56^{b}	106 ± 13	547 ± 107	234 ± 49^{ab}	1203 ± 108	332 ± 54	385 ± 85	106 ± 23	96 ± 9
April (Mature)	12.69 ± 2.22^{c}	119 ± 21	412 ± 109	$173 \pm 28^{a,d}$	1952 ± 137	374 ± 52	375 ± 101	178 ± 56	109 ± 27
				Acclimation temperature: 18°					
September (Regressed)	0.99 ± 0.18	127 ± 17	598 ± 145	512 ± 83^{d}	1273 ± 174	330 ± 50	375 ± 36	45 ± 6^{d}	145 ± 32^{d}
April (Mature)	13.74 ± 2.12^{e}	94 ± 19	578 ± 71	375 ± 61^{e}	1260 ± 102	324 ± 60	388 ± 59	95 ± 18^{e}	67 ± 13^{e}

Note. Values represent means ± SEM; n = 5-7/group. Statistically significant differences at $p < 0.05$ are indicated by superscripts: *a* different from b and c, *ab* not different from *a* or *b* (Duncan's multiple range test); *d* different from e (Student's *t* test).

* Values given are GnRH content per brain fragment which are normalized to 25 g gonad-free body wt fish (as described under Section 2.2, Materials and Methods).

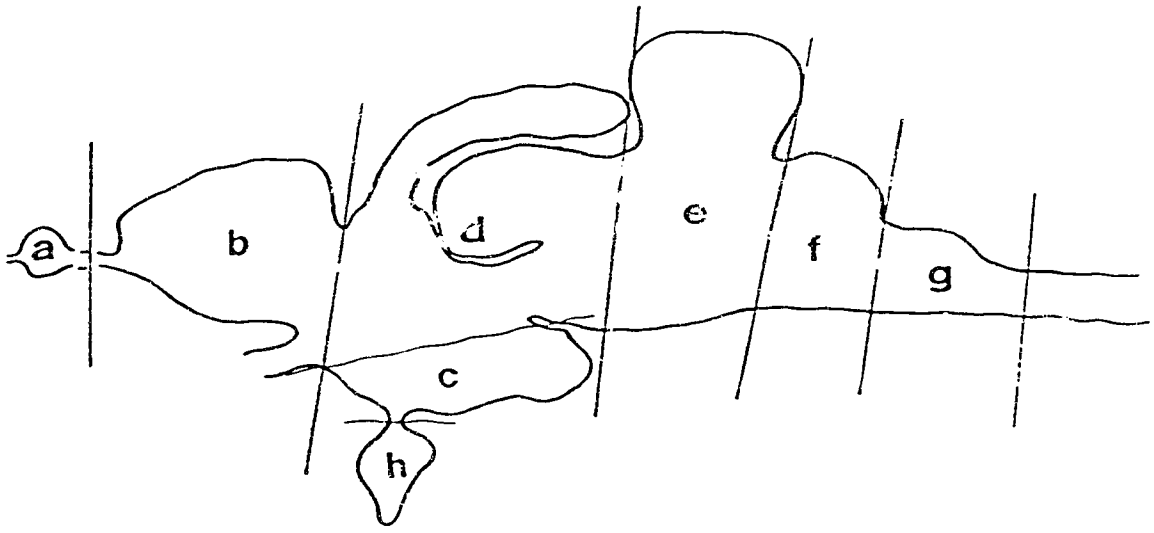

Fig. 2.1. Diagrammatic sagittal section of goldfish brain showing the discrete brain areas dissected for the determination of immunoreactive GnRH. Letters represent the following brain areas: a, olfactory bulbs and tracts; b, telencephalon, including optic nerve and preoptic region; c, hypothalamus; d, optic tectum-thalamus, including anterior part of cerebellum; e, cerebellum; f, medulla; g, spinal cord (0.5 cm), and h, pituitary.

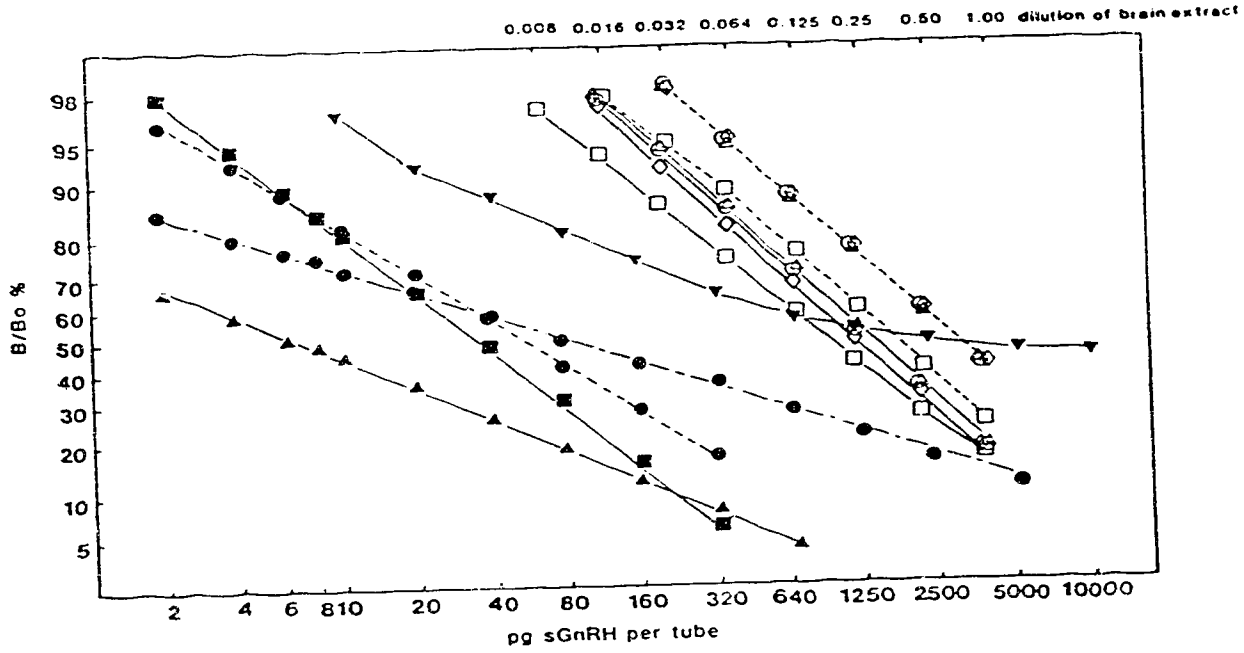

Fig. 2.2. Inhibition of binding of ^{125}I-sGnRH to the antiserum by the sGnRH standard (—■—), cGnRH-I (—▲—), mGnRH (—●—), cGnRH-II (—●—), lamprey GnRH (—▼—) and acid extracts of olfactory bulbs (—△—), telencephalon (—□—), hypothalamus (—△—), optic tectum-thalamus (—○—), cerebellum (—◇—), medulla(—□—), spinal cord (—◇—) and pituitary (—○—) of female goldfish. Each point represents the average of duplicate determinations.

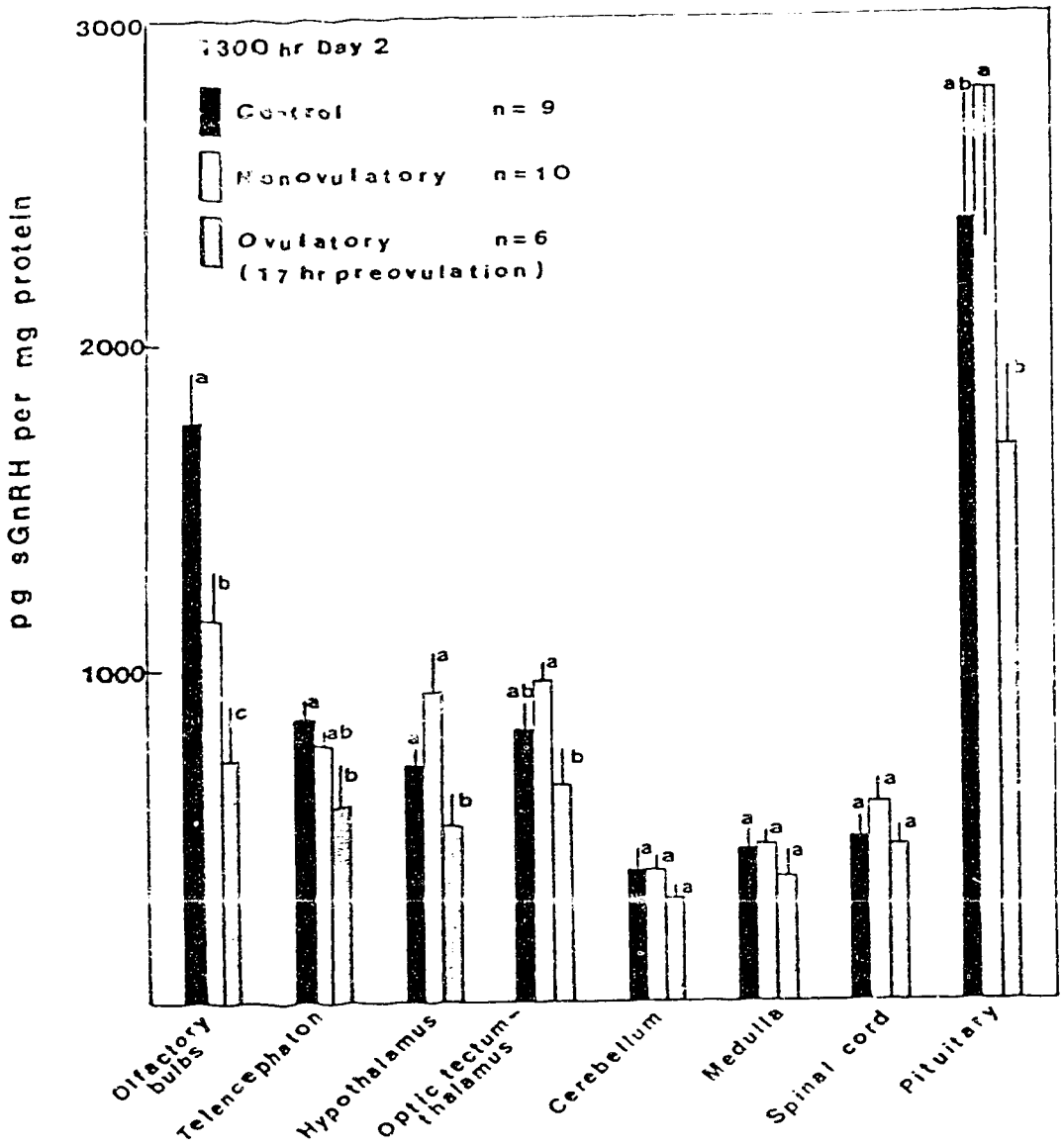

Fig. 2.3. GnRH concentrations in discrete brain areas of preovulatory goldfish (experiment II). Statistical comparisons were made by one-way analysis of variance followed by Duncan's mutiple range test. Means without a common letter are different at the 5% level of probability.

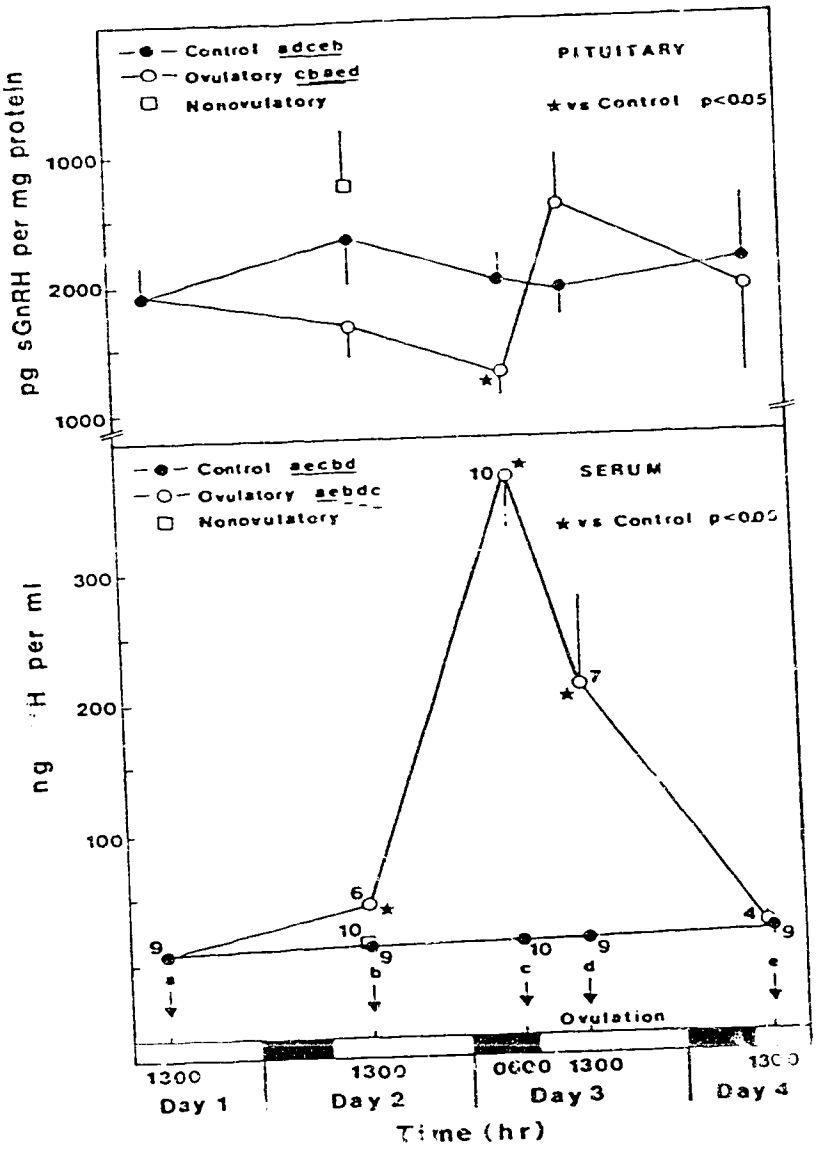

Fig. 2.4. Temporal changes in pituitary GnRH concentrations and serum GtH level at various times during periovulatory period (experiment II). Points represent mean ± SEM. The number of fish in each group is shown by the number adjacent to the point in the lower panel showing changes in serum GtH levels. Sexually mature female goldfish were kept on a 16 L:8 D photoperiod (darkened horizontal bars represent dark period). The dotted horizontal bar indicates the time period over which ovulation occurred. The sampling times are indicated by arrows and letters: a 1300 hr Day 1, b 1300 hr Day 2, c 0600 hr Day 3, d 1300 hr Day 3 and e 1300 hr Day 4. The unpaired Student's t test using log-transformed data was used to compare values at each sampling time. Analysis of variance and Duncan's mutiple range test were used to test for significance in the changes in GnRH concentration within control and experimental groups for each brain area. Underlined letters represent means which are statistically equivalent ($p < 0.05$).

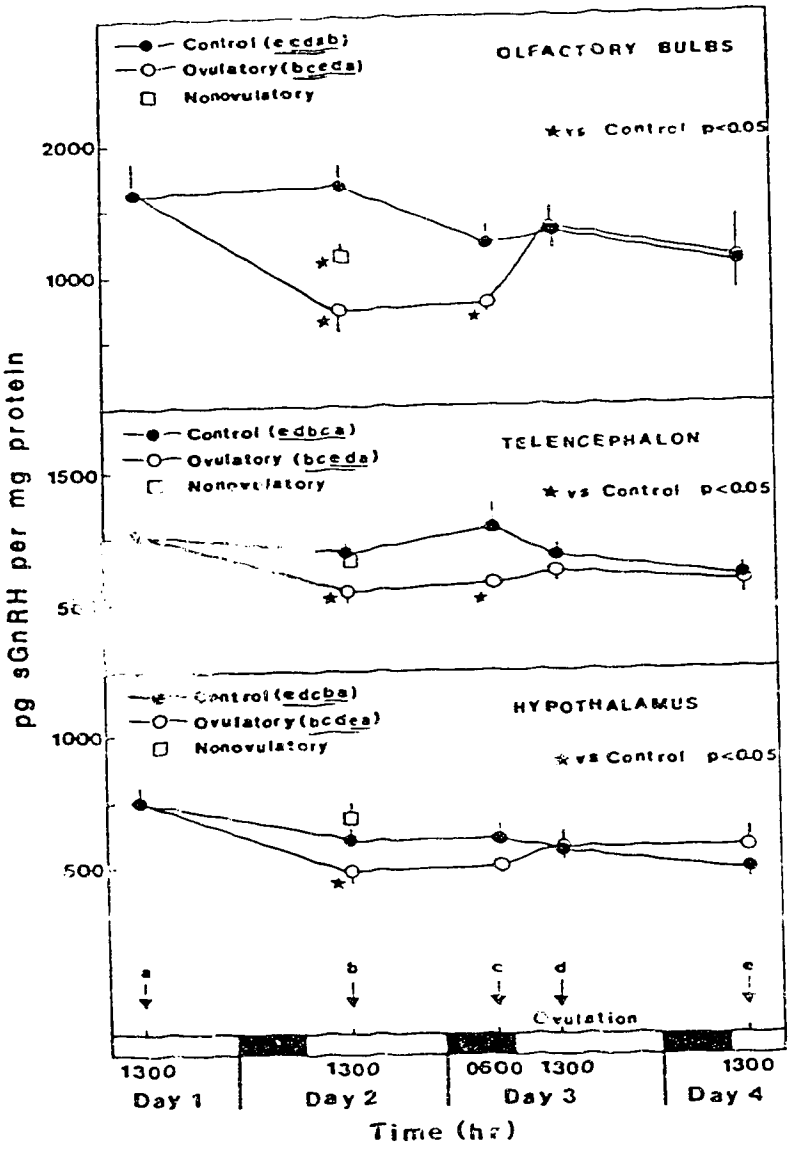

Fig. 2.5. Temporal changes in GnRH concentrations in selected brain areas at various times during periovulatory period (experiment II). Explanations and abbreviations are the same as those of Fig. 2.4.

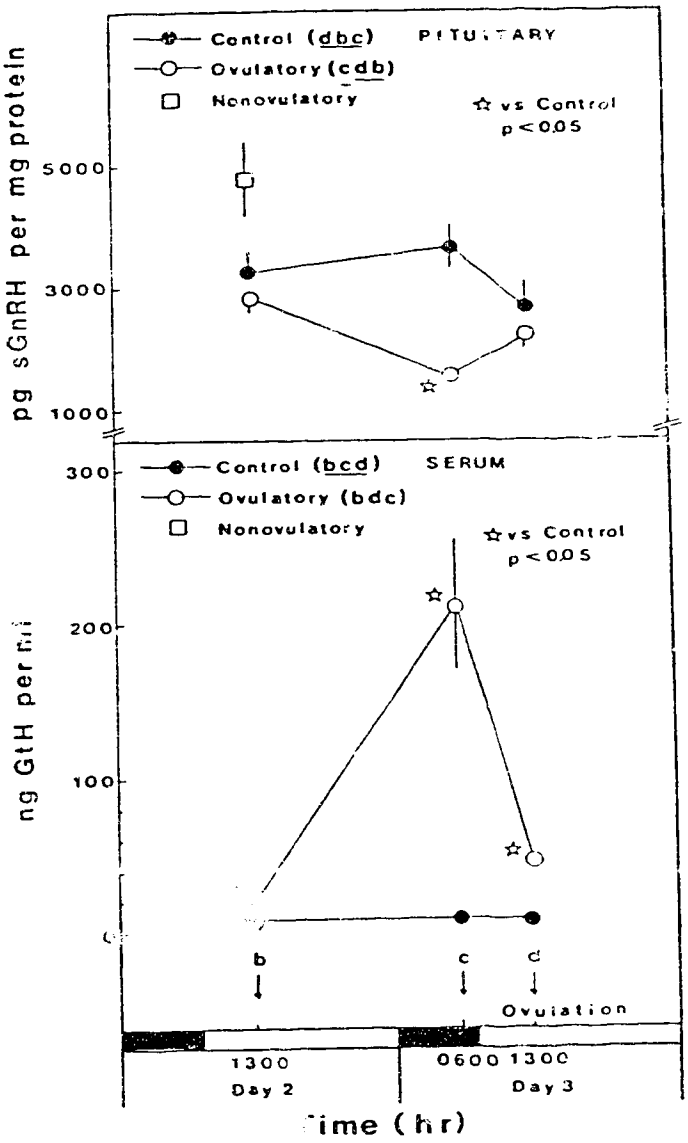

Fig. 2.6. Periovulatory changes in serum GtH level and pituitary GnRH concentrations (Experiment III). Explanations and abbreviations are the same as those of Fig. 2.4.

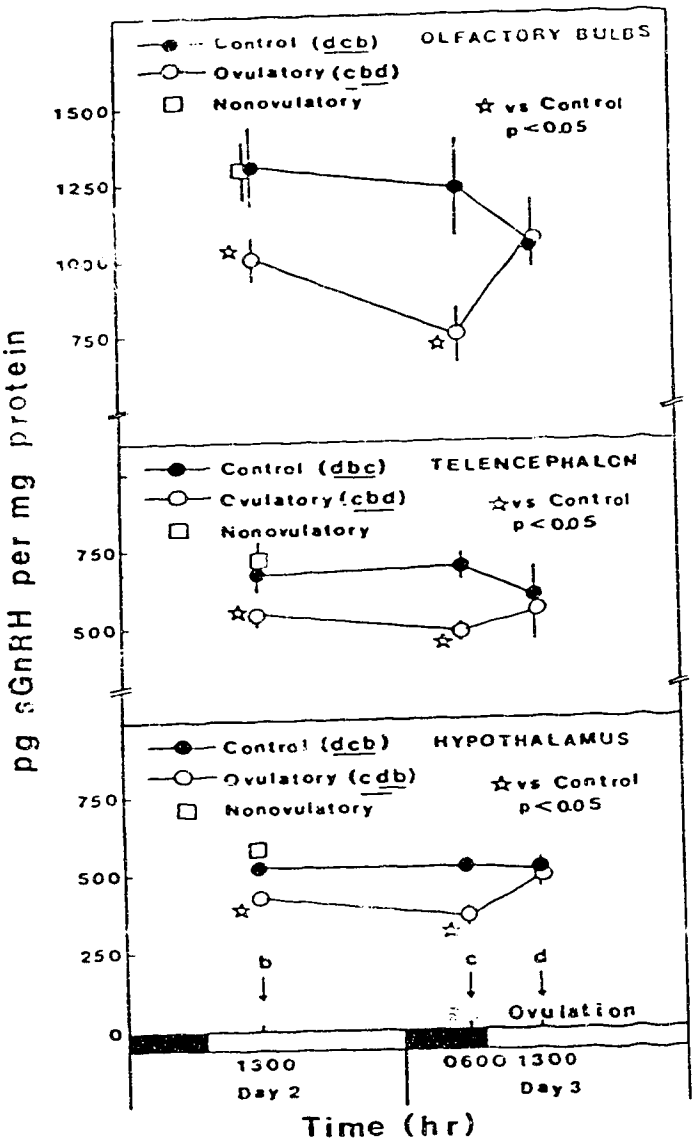

Fig. 2.7. Periovulatory changes in GnRH concentrations in olfactory bulbs, telencephalon, and hypothalamus (Experiment III). Explanations and abbreviations are the same as those of Fig. 2.4.

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3. ALTERATIONS IN GONADOTROPIN-RELEASING HORMONE IMMUNOACTIVITIES IN DISCRETE BRAIN AREAS OF MALE GOLDFISH DURING SPAWNING BEHAVIOR [1]

3.1 INTRODUCTION

Activation of gonadotropin (GtH) and androgen secretion during exposure to sexual stimuli has been demonstrated in males of various vertebrates (Dufty and Wingfield, 1986; Gartska and Crews, 1982; Harding, 1981; O'Connell *et al.*, 1981). In rats, there is strong evidence to suggest that gonadotropin-releasing hormone (GnRH) is involved in both endocrine and behavioral changes during reproduction (Moss and Dudley, 1984). GnRH has also been demonstrated to facilitate reproductive behavior in other vertebrate species, including the vole (Boyd and Moore, 1985), mouse (Moss and Dudley, 1984), pigeon (Cheng, 1977), lizard (Alderete *et al.*, 1980), frog (Kelley, 1982), and newt (Moore *et al.*, 1987); direct evidence for the involvement of GnRH in reproductive behavior of teleosts, however, is lacking.

In male goldfish, interaction with females which were induced to perform spawning behavior by intramuscular injection of prostaglandin (PG) F2α rapidly elevates serum GtH levels (Kyle *et al.*, 1983). Pheromonal input for the behavioral responses of male goldfish to PG-treated females has been demonstrated (Sorensen *et al.*, 1986). Sectioning of the medial olfactory tract pathways abolished the behavioral responses to PG-treated females (Stacey and Kyle, 1983). Exposure to water-borne pheromones from

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PG-treated females stimulates male spawning behavior (Sorensen *et al.*, 1986, 1988). The GtH response of the male goldfish to PG-treated females, however, depends on both pheromonal activation of spawning behavior and subsequent behavioral interaction with the females (Kyle *et al.*, 1983; Sorensen *et al.*, 1989). To [illegible] the central mechanisms responsible for the behavioral and endocrine responses [illegible] goldfish exposed to PG-treated females have not been elucidated. To study the [illegible]sible involvement of the GnRH neuronal system during spawning behavior in male [illegible]ish, the effects of spawning with PG-treated females on brain GnRH and serum GtH levels were investigated. In addition, the involvement of medial olfactory tract pathways in mediating both brain GnRH and serum GtH responses to spawning behavior was also demonstrated.

3.2 MATERIALS AND METHODS

Experimental animals.

Goldfish, 20 - 35 g body weight, were purchased from Grassyfork Fisheries Co. (Martinsville, IN), at various times of year. The fish were held in 3600-liter flow-through aquarium at 15 ± 1 °C on a simulated natural (Edmonton) photoperiod for 1 to 2 weeks after arrival in the laboratory. In all experiments, mature males having expressible milt were used.

Temporal changes in hormone levels during spawning behavior.

Eighty sexually mature male goldfish were equally divided among four 180-liter flow-through aquaria and acclimated to 16 hours light and 8 hours darkness at 15°C for two weeks. Three days before the experiment, fish were warmed to 20°C and the night before the experiment, artificial floating vegetation was added to all aquaria to provide a spawning substrate. Sexual receptivity in females was induced by intramuscular injection

of $PGF_2\alpha$ (5 μg per fish; Stacey, 1976), and at the beginning of the photophase, normal males or sexually receptive females (20 per aquarium) were introduced into the two control and two treatment aquaria respectively. The spawning behavior of male goldfish in response to PG-treated females has been described elsewhere (Kyle and Peter, 1982). Introduced fish were distinguished from experimental fish by metal tags attached to the operculum. At -1, 1, 2, 4 and 6 hr relative to the onset of exposure, experimental male fish were sampled randomly from each control and treatment groups for blood and brain as described in Chapter 2. Briefly, blood was sampled from the caudal vasculature after fish were anesthetized in 0.05% tricaine methanesulfonate (Syndel, Vancourver, BC). Brain and pituitary were removed immediately after blood sampling. Olfactory bulbs (including part of the olfactory nerves and olfactory tracts, attached rostrally and caudally respectively), telencephalon (including preoptic region) and hypothalamus (including inferior lobes) were dissected from the brain (see Chapter 2). The pituitary and each brain area were immersed in 2 ml of ice-cold 2N acetic acid and extracted for GnRH. Serum collected from blood was frozen on dry ice and stored at -30°C until radioimmunoassay (RIA) for GtH. The experiment was repeated with n=24 per control and treatment group. Data from the two experiments were pooled for statistical analysis.

Effects of sectioning the olfactory tracts on hormonal changes during spawning behavior.

Surgical operation to remove olfactory tracts (OTs) was performed as described by Stacey and Kyle (1983). Briefly, a three-sided bone flap above the olfactory tracts was cut with a dental saw and deflected to expose the OTs. One of three types of bilateral olfactory tract sectioning was performed by using iris scissors and the cut OTs were removed by gentle aspiration. For medial olfactory tract section (mOTX) and lateral olfactory tract section (lOTX), only medial OTs and lateral OTs were removed respectively. For olfactory tract section (OTX), both medial and lateral OTs were cut. In sham-operated (SHAM) fish,

the OTs were not cut. The cranial cavity was then filled with physiological saline and the bone flap was put back in place to close the skull. Three days after surgery, groups of OTX, mOTX, lOTX, or SHAM fish were exposed either to nothing (control) or to PG-injected females in separate 180 liter aquaria. At 2 hour after the onset of exposure, serum was sampled, the fish killed, the surgical operation on the olfactory tracts confirmed and the brain removed as described previously.

Radioimmunoassays for GtH and GnRH.

Serum GtH levels were determined by a RIA for carp GtH as described previously (Peter *et al.*, 1984). Total GnRH immunoactivity in each brain area was measured using a double antibody radioimmunoassay (RIA) with ^{125}I-[Trp^7, Leu^8]-GnRH (salmon GnRH or sGnRH) as tracer and synthetic sGnRH (Peninsula Laboratories, San Carlos, CA) as standards similar to that described in Chapter 2, with slight modification of the procedure for iodination and purification of tracer. Briefly, sGnRH (5 µg) was iodinated by incubation with 5 µg Chloramine T for 2 min. ^{125}I- sGnRH was purified using a carboxymethyl cellulose (fine mesh; Sigma) cation exchange column (0.6 x 6 cm). The column was eluted with 15 ml 2 mM ammonium acetate buffer (pH 4.5) and 90 ml 100 mM ammonium acetate buffer (pH 4.5) at at flow rate of 0.75 ml per min. This RIA using antiserum PBL-49, recognizes both sGnRH-like and [His^5, Trp^7, Tyr^8]-GnRH (chicken GnRH-II or cGnRH-II)-like molecules in various parts of the goldfish brain (in Chapter 8). cGnRH-II (synthesized by R. Millar) showed 32.6% crossreactivity in this RIA with synthetic sGnRH (Sigma) used as standards. Total protein content of each brain area was determined by Bradford's method (Bio-Rad).

Statistical analysis.

The data were analyzed using the Student's t test, or by one-way analysis of variance and Duncan's multiple range test.

3.3 RESULTS

Temporal changes in hormone levels during spawning behavior.

Introduction of PG-treated females, but not of untreated males, into the aquaria stimulated spawning behavior in male goldfish for at least four hours. As shown in Fig. 3.1, male goldfish exposed to other males did not show significant changes in serum GtH levels or GnRH levels in various brain areas during the test period. Male goldfish exposed to PG-treated females showed significant increases in GnRH concentrations in the olfactory bulbs, telencephalon and hypothalamus at 1 and 2 hr following the onset of exposure compared to controls (males exposed to untreated males). Pituitary GnRH concentrations did not show significant changes between control and treatment groups at any of the sampling times or between various sampling times within each group. GnRH concentrations of each brain area returned to pre-exposure levels by four hours after exposure to PG-treated females. Serum GtH levels showed significant increases at 1, 2 and 4 hr after exposure to PG-treated females; serum GtH returned to a level above control values six hours following the exposure.

Effects of sectioning the olfactory tracts on hormonal changes during spawning behavior.

Three days after surgery, spawning behavior by either OTX or mOTX male goldfish was not observed during exposure to PG-treated females, as reported previously (Stacey and Kyle, 1983). In contrast, normal spawning behavior by lOTX fish was observed in response to PG-treated females, similar to sham-operated male fish. As shown in Fig. 3.2, mOTX resulted in a significant increase in the GnRH concentrations in the olfactory bulbs compared to sham-operated fish; OTX and mOTX resulted in a significant decrease in the GnRH concentrations in the telencephalon. None of the surgical treatments (OTX, mOTX and lOTX) changed pre-exposure serum GtH levels. Sham operated males

exposed to PG-treated females showed significant increases in GnRH concentrations in the olfactory bulbs, telencephalon and hypothalamus; similar changes were observed in lOTX males exposed to PG-treated females. However, OTX and mOTX males exposed to PG-treated females had no changes in GnRH concentrations in the various brain areas. None of the surgical treatments or the exposure to PG-treated females caused any changes in pituitary GnRH levels. Serum GtH levels increased in sham operated and lOTX fish but not in OTX or mOTX fish, two hours after exposure to PG-treated females.

3.4 DISCUSSION

The present study demonstrates that when male goldfish are exposed to PG-treated females, GnRH levels in the olfactory bulbs, telencephalon and hypothalamus are increased significantly for one to two hours. The pituitary GnRH levels, however, did not show significant changes. Exposure of male goldfish to other males did not result in changes in brain GnRH concentrations within the experimental period, indicating the specificity of the hormonal responses to exposure to the sexually receptive PG-treated females. The time-course of changes in the GnRH concentrations in the olfactory bulbs, telencephalon and hypothalamus correlates well with the observed time course of increases in serum GtH levels. However, the lack of significant changes in the GnRH levels in the pituitary during spawning behavior indicates that pituitary GnRH may not be causally related to the increases in serum GtH levels. Since pituitary GtH secretion in goldfish is known to be regulated by both a stimulatory GnRH and an inhibitory dopaminergic system (Peter *et al.*, 1986), a likely alternative explanation for the GtH response during spawning behavior would be withdrawal of dopaminergic inhibition. However, it is also possible that the amount of GnRH being released from the nerve terminals in the pituitary was replenished by synthesis of the neuropeptide. This latter interpretation is supported by the results in

this study showing that males exposed to PG-females have elevated GnRH levels in the brain areas (olfactory bulbs, telencephalon) which have been shown to contain GnRH cell bodies (Kah *et al.*, 1986).

In Chapter 2, significant decreases in GnRH concentrations in the olfactory bulbs, telencephalon, hypothalamus and pituitary were coincident with the ovulatory surge in serum GtH levels in female goldfish. This contrasts with the present findings of an increase in GnRH concentrations in the olfactory bulbs, telencephalon and hypothalamus coincident with the increased serum GtH levels in male goldfish exposed to PG-treated females. Notably, the magnitude of the increases in serum GtH levels during the ovulatory surge are much greater than those observed in males exposed to PG-treated females. Presumably the ovulatory surge of GtH requires a surge in release of GnRH that is not compensated by synthesis until after the surge is over (Chapter 2). On the other hand, exposure of male goldfish to PG-treated females likely has a stimulatory effect on the synthesis of the GnRH in the brain through combined pheromonal and behavioral inputs to the GnRH neuronal system (see later discussion), without a concomitant major release of GnRH in the pituitary, resulting in a net increase in brain GnRH levels.

The alterations in brain GnRH levels in male goldfish during exposure to PG-treated females could be accounted for by at least two mechanisms: pheromonal activation of the brain GnRH neuronal system, and/or indirect stimulation as a result of the behavioral interactions. Pheromonal input through the medial olfactory tract system has been demonstrated to mediate the behavioral responses of male goldfish to PG-treated females (Sorensen *et al.*, 1986; Stacey and Kyle, 1983). The pheromones released from PG-treated females, PG pheromones, may be a mixture of PGF2α and its metabolite, 15-keto-prostaglandin F2α (15-keto-PGF2α; Sorensen *et al.*, 1988). The GtH response of the male goldfish to PG-treated females, however, apparently depends on both exposure to PG pheromones and subsequent behavioral interaction with conspecifics. For example, serum GtH levels are not increased in isolated males exposed to water-borne PGFs (PGF2α and

15-keto-PGF2α) or the odor of PG-treated females (Kyle *et al.*, 1983; Sorensen *et al.*, 1989); however, water-borne PGFs induce courtship behavior in grouped males, as well as an increase in their serum GtH levels (Sorensen *et al.*, 1989). It remains to be studied whether changes in brain GnRH levels are related to the intensity or duration of spawning activities in male goldfish. It was hypothesized that behaviorally stimulated GtH release in male goldfish may act in a feed-forward manner to facilitate subsequent spawning activities in the spawning season (Kyle *et al.*, 1983). This hypothesis is consistent with the finding that male goldfish allowed to spawn with sexually receptive females daily for 20 days showed gradual and marked increases in serum GtH levels (Stacey, 1987).

The alteration of GnRH concentrations in the olfactory bulbs of male goldfish during spawning behavior suggests a role of GnRH in these structures in the associated hormonal changes. Although it has been shown that the medial olfactory tracts are important in mediating behavioral and hormonal changes to pheromonal stimuli in male goldfish (Kyle *et al.*, 1987), it is controversial whether this implicates the olfactory nerve or the nervus terminalis (terminal nerve, TN). One hypothesis suggests that the GnRH-containing TN directly mediates behavioral and/or endocrine responses to sex pheromones (Demski and Northcutt, 1983; Wirsig and Leonard, 1987). Since the GnRH neurons in the TN innervate the olfactory lamellae (Kyle and Stell, 1988), retina and forebrain areas (von Bartheld and Meyer, 1986; Stell and Walker, 1987), the altered GnRH neuronal activities in the TN may constitute part of an integrated mechanism through which olfactory and visual components of the response to sex pheromones are coordinated. Studies in female newts tentatively support this hypothesis; Moore *et al* (1987) reported that GnRH content in the TN-containing anterior telencephalon, but not those in other hypothalamic areas, was elevated in females during sexual behavior with males. However, using electro-physiological techniques, recent studies in male goldfish failed to detect any changes in activity of TN during exposure of the olfactory organ to sex pheromones (including PGFs;

Fujita *et al.*, 1989). On the other hand, responses of TN to mechanical stimulation of the body led Fujita *et al* (1989) to postulate that TN is sensitive to the frequent tactile stimulation that occurs during spawning behavior of male goldfish. The possibility that brain GnRH is not only influenced by pheromonal signals through olfactory pathways but also by tactile stimulation during spawning behavior in male goldfish remains to be explored.

Alternatively, GnRH neurons not associated with the TN could receive pheromonal inputs indirectly through interactions with the secondary olfactory fibers (mitral cell axons); the terminal fields of the secondary olfactory fibers overlap those of the terminal nerve in the ventral telencephalon and the anterior preoptic areas (Levine and Dethier, 1985; von Bartheld *et al,* 1984). Since the ventral telencephalon and the anterior preoptic areas are known to be important in the control of spawning behavior (Koyama *et al.*, 1984, 1985; Kyle and Peter, 1982) and pituitary GtH secretion (Peter et al., 1986) respectively, interactions of GnRH neurons with the processed pheromonal signals at these two brain areas would allow coordinated activation of spawning behavior, brain GnRH activity and pituitary GtH secretion.

There are several other examples from studies on mammals of changes in concentrations of GnRH in the olfactory bulbs in response to external stimuli. Exposure of male mice to other males or ovariectomized females resulted in a significant increase in GnRH concentrations in olfactory bulbs (Dluzen and Ramirez, 1983). In female prairie voles, GnRH concentrations in olfactory bulbs showed significant increases following exposure to male urine (Dluzen and Ramirez, 1981). Although these studies demonstrated the ability of external stimuli to influence GnRH levels in the olfactory bulbs, a consistent relationship between the serum LH levels and GnRH concentrations in the olfactory bulbs has not been obtained. It should be noted, however, that these studies described transient elevations of GnRH levels in the olfactory bulb areas in response to external stimuli

without apparent (or prolonged) behavioral interaction, which is, in contrast, a necessary condition for the increases in brain GnRH and serum GtH levels in male goldfish exposed to PG-treated females. These observations in mammals are analogous to those in male goldfish in which pituitary GtH secretion is stimulated by the exposure to 17α, 20β-dihydroxy-4-pregnen-3-one (17,20βP), a "primer" sex pheromone released from preovulatory goldfish (Dulka *et al.*, 1987). In contrast to the PG pheromone, water-borne 17,20βP induces increases in serum GtH levels in male goldfish without apparent requirement for behavioral interaction with the females (Sorensen *et al.*, 1989). The central mechanisms for the stimulation of pituitary GtH secretion in male goldfish by 17,20βP, however, have not yet been investigated.

In summary, the present study demonstrates an alteration in brain GnRH and serum GtH levels during spawning behavior in male goldfish. Pheromonal input via the medial olfactory tract pathways for the activation of behavioral and endocrine changes in response to PG-treated females was also indicated. It is hypothesized that concomitant changes in GnRH concentrations in different brain areas may constitute part of an integrated mechanism to initiate and/or sustain the sequence of behavioral and endocrine changes during spawning behavior in male goldfish. Further studies are needed to elucidate the precise anatomical pathways and neurochemical mechanisms by which the pheromonal signals from PG-treated females are transmitted and transformed to influence the behavior and endocrine functions of male goldfish.

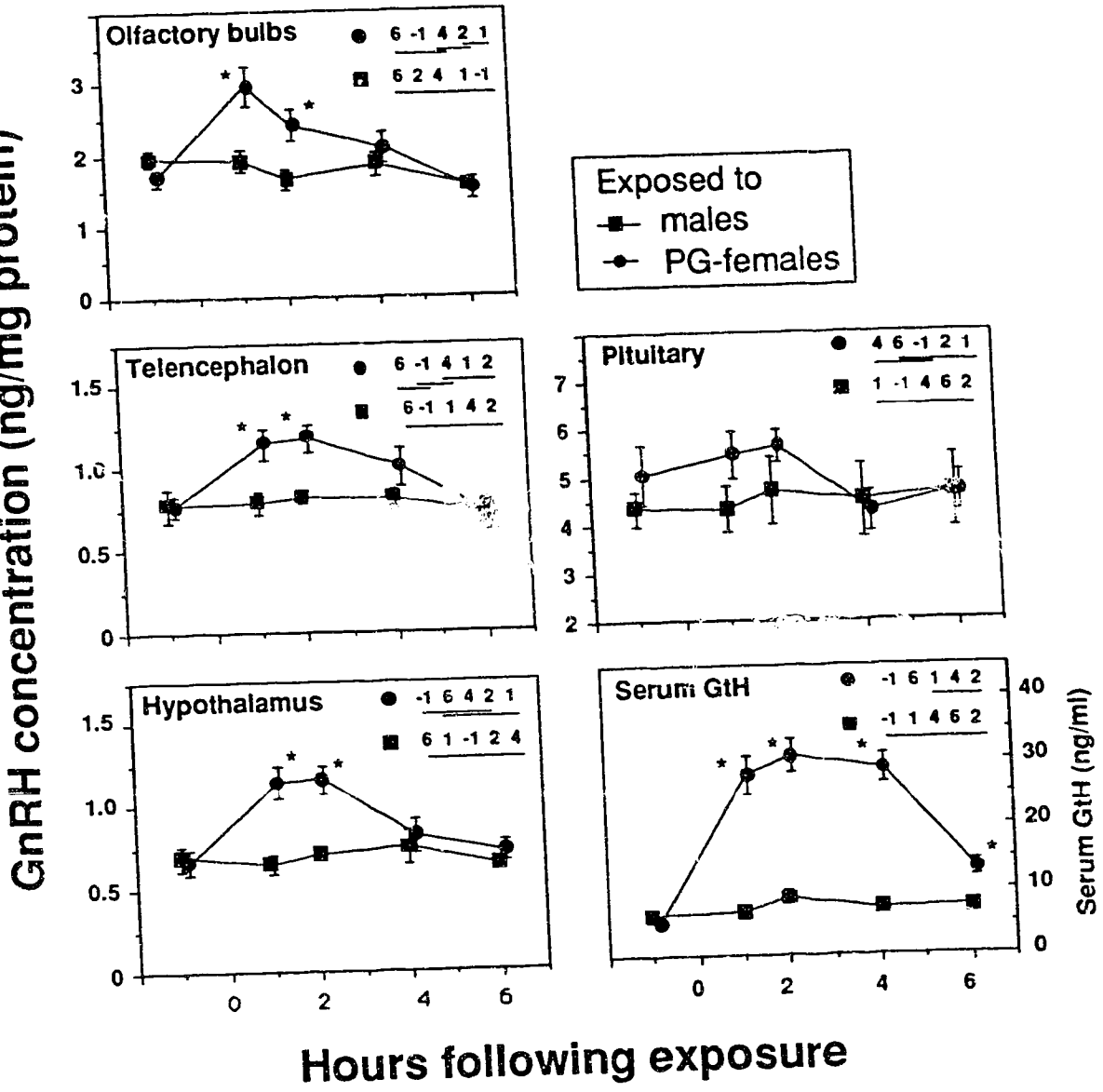

Fig. 3.1. Temporal changes in serum GtH levels, and GnRH concentrations in discrete brain areas of male goldfish during exposure to either normal males or sexually receptive females. Values represent mean ± SEM, n=11-12. Exposure started at the beginning of the photophase (16L/8D), and fish were sampled at -1, 1, 2, 4 and 6 hr relative to the onset of exposure . Sexually receptive females were induced by intramuscular injection of PGF2α (5 ug per fish). * indicates a significant ($p < 0.05$, unpaired Student's t-test) difference from the control (exposure to males) groups. Within the control groups and treatment (exposure to PG-treated females) group, the sampling times underlined by the same line represent means which are not statistically different (ANOVA and Duncan's multiple range test, $p < 0.05$)

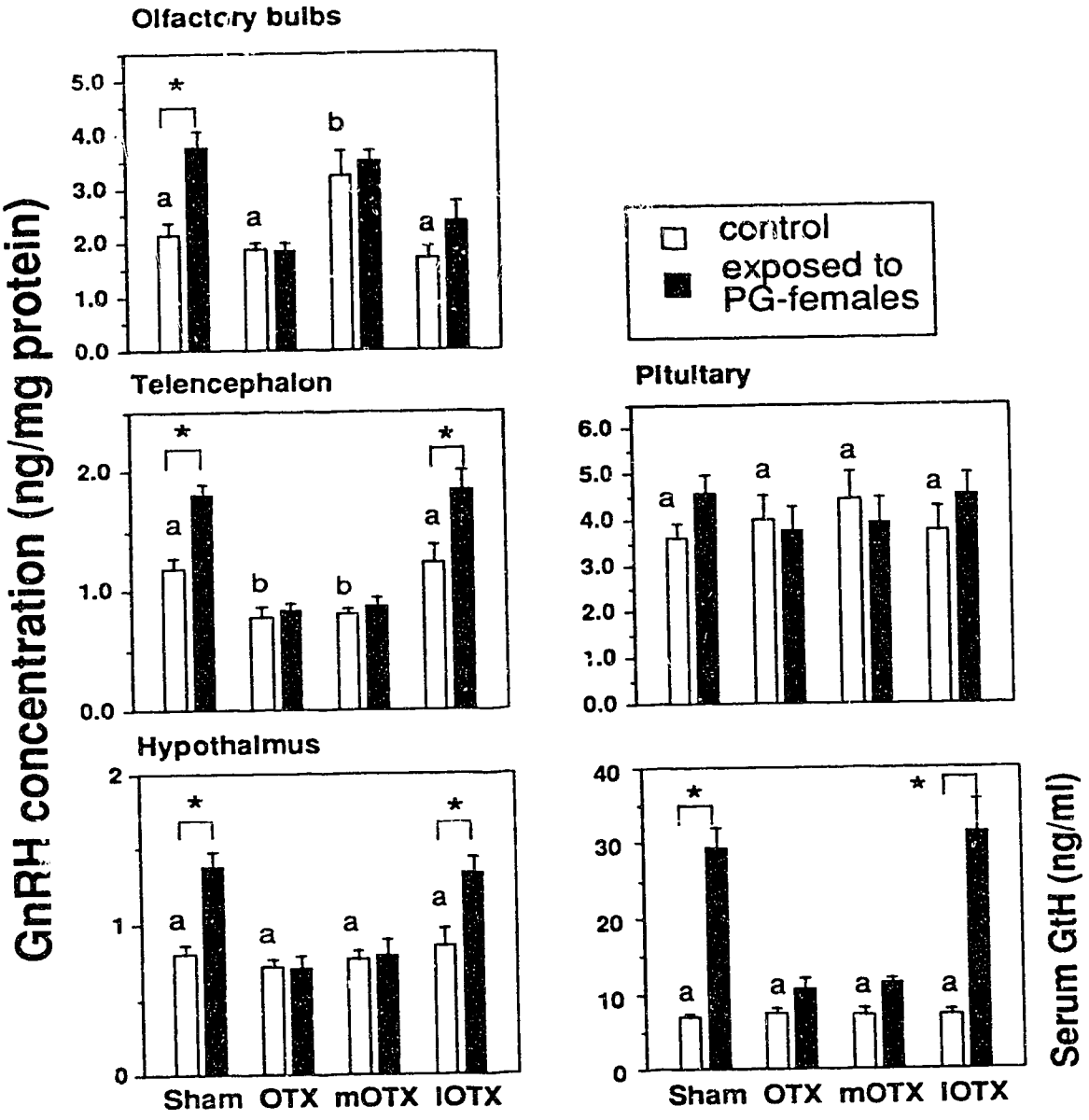

Fig. 3.2. Serum GtH levels and GnRH concentrations in discrete brain areas of male goldfish not exposed to (control) or exposed to PG-treated females for two hours 3 days after bilateral olfactory tract section (OTX, n=8), medial olfactory tract section (mOTX, n=16), lateral olfactory tract section (lOTX, n=8) or a sham operation (SHAM, n=16). Each column represents mean ± SEM. * indicates a significant difference ($p < 0.05$, unpaired Student's t-test) from the respective control group. Comparisons of the means among control groups of Sham, OTX, mOTX and lOTX were made by ANOVA and Duncan's multiple range test. Means of the control groups without a common letter are significantly different at the 5% level of probability.

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4. DOPAMINERGIC REGULATION OF BRAIN GONADOTROPIN-RELEASING HORMONE IN MALE SPAWNING BEHAVIOR [1]

4.1 INTRODUCTION

In teleosts, considerable evidence shows that gonadotropin secretion is regulated by the dual effects of gonadotropin-releasing hormone (GnRH) and dopamine which inhibits GnRH-stimulated GtH release (de Leeuw *et al.*, 1987; Peter *et al.*, 1986). It is not known, however, whether dopamine also influences the activities of brain GnRH neurons. An interaction between the central dopaminergic and GnRH peptidergic systems is suggested by the anatomical proximity of the preoptic-hypophyseal dopaminergic (Kah *et al.*, 1987) and GnRH peptidergic (Kah *et al.*, 1986) pathways in goldfish. In this study, the effects of the centrally active dopaminergic agonist apomorphine and antagonist pimozide on GnRH levels in pituitary and discrete brain areas were investigated. In Chapter 3, it was shown that spawning stimuli from prostaglandin-treated females (PG-females) stimulate transient increases in brain total GnRH levels and serum GtH levels. In this study, the physiological consequences of dopaminergic receptor stimulation or blockade on the responses of brain GnRH and serum GtH levels to spawning stimuli from PG-females were investigated.

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4.2 MATERIALS AND METHODS

General.

Goldfish, 20 - 35 g body weight, were purchased from Grassyfork Fisheries Co. (Martinsville, IN). The fish were acclimated in 3600-liter flow-through aquarium at 15 ± 1°C on a simulated natural (Edmonton) photoperiod for 1 to 2 weeks after arrival in the laboratory.

Apomorphine ((±)apomorphine HCl) was purchased from Sigma Chemical Company, St. Louis, Missouri. Pimozide was a gift from Jansen Pharmaceutica Ltd., Beerse, Belgium. All drugs were made up in a vehicle of dimethylsulfoxide (DMSO): propylene glycol: acidified physiological saline in a volume ratio of 2:48:50. All drugs were injected intraperitoneally on a nanomole (nmol) per gram body weight basis. Control groups were given an equivalent volume of vehicle (5 µl per gram body weight).

Fish were anesthetized by immersion in 0.05% tricaine methanesulfonate (Syndel, Vancouver, BC) before handling. Prior to Experiments 1, 2 and 3, fish were weighed and identified with a numbered metal tag attached to the operculum. Blood samples were taken by puncturing the caudal vasculature with a 25-gauge needle attached to a one ml syringe. Serum collected after centrifugation of blood was frozen on dry ice and stored at -30°C until radioimmunoassay (RIA) for GtH. Brain and pituitary were removed immediately after blood sampling as described previously (Chapter 2). Olfactory bulbs (including part of the olfactory nerves and olfactory tracts, attached rostrally and caudally, respectively), telencephalon (including preoptic region) and hypothalamus (including inferior lobes) were dissected from the brain. Pituitary and each brain area were immersed in 2 ml of ice-cold 2N acetic acid and extracted for GnRH by sonication and centrifugation as described previously (Chapter 2). Lyophilized brain extracts were reconstituted in assay buffer and assayed for total GnRH immunoactivities.

Effects of pimozide and apomorphine on levels of brain GnRH and serum GtH levels in normal male fish.

Experiment 1 - Effects of different doses of pimozide. Male goldfish (n=8-10 per group) at early stages of gonadal recrudescence (gonadosomatic index, GSI =2.4 ± 0.3%; mean ± SEM) were used. Pimozide, in doses ranging from 0.1 to 20 nmol/g body weight, was injected intraperitoneally. Blood and brain samples were collected 24 hr after injection.

Experiment 2 - Time-course effects of pimozide. Male goldfish (n=8 per group) at mid-stages of gonadal recrudescence (GSI= 3.8 ± 0.5%) were treated with either vehicle or pimozide (10 nmol/g BWt) , and sampled at 3, 9 and 15 hr after treatment

Experiment 3 - Effects of pimozide and apomorphine. Male goldfish (n=9-10 per group) at mid-stages of gonadal recrudescence (GSI = 3.6 ± 0.4%) were used. Pimozide, apomorphine or a combination of pimozide and apomorphine were injected at a dose of 1 nmol/g BWt. Blood and brain samples were taken 24 hr after injection.

Effects of apomorphine and pimozide on levels of brain GnRH and serum GtH in behaviorally stimulated male fish

Experiments 4 and 5 - In each experiment, four groups of spermiating male goldfish (n = 30 per group) were acclimated to 16 hours light and 8 hours darkness at 15°C for two weeks in 225-liter flow-through aquaria. Three days before the experiment, male fish were warmed from 15°C to 20°C and at 24 hr before beginning the experiment, artificial floating vegetation was added into all aquaria to provide a spawning substrate. Fish were sampled for blood and brains at -1, 2 and 6 hour relative to the onset of exposure to other males (control) or PG-females. As described previously, female goldfish induced to be sexually receptive by intramuscular injection of $PGF_2\alpha$ (5 µg per fish) will perform spawning behavior with males (Stacey, 1976). The four groups of fish were given one of

the following treatments in Experiments 4 and 5:

(i) Vehicle pretreatment and exposure to other males;

(ii) Apomorphine (Experiment 4) or pimozide pretreatment (Experiment 5) and exposure to other males;

(iii) Vehicle pretreatment and exposure to PG-females;

(iv) Apomorphine (Experiment 4) or pimozide pretreatment (Experiment 5), and exposure to PG-females;

Males or PG-females (n=20) were introduced into the aquaria at 2 hr after the beginning of the dark phase. Introduced fish were distinguished from experimental male fish by metal tags attached to the operculum. In Experiment 4, experimental fish were injected intraperitoneally with either vehicle or apomorphine (33 nmol/g BWt) 5 hr before the onset of exposure to either normal males or PG-females. In Experiment 5, experimental fish were injected intraperitoneally with either vehicle or pimozide (10 nmol/g BWt) 12 hr before the onset of exposure to either normal males or PG-treated females.

Radioimmunoassays for GtH and GnRH.

Serum GtH levels were determined by a RIA for carp maturational GtH as described previously (Peter *et al.*, 1984). In goldfish brain, pituitary (Chapter 8) and serum (Peter *et al.*, 1989), two forms of GnRH immunoactivities have been characterized as salmon GnRH ([Trp^7, Leu^8]-GnRH, sGnRH) and chicken GnRH-II ([His^5, Trp^7, Tyr^8]-GnRH, cGnRH-II) using a HPLC system and radioimmunoassay (RIA) with different GnRH antisera.Total GnRH immunoactivity in each brain area was measured using a double antibody RIA with ^{125}I-salmon GnRH (sGnRH, [Trp^7, Leu^8]-GnRH) as tracer and synthetic sGnRH (Peninsula Laboratories, San Carlos, CA) as standards (see Chapters 2 and 3). As reported in Chapter 3, this RIA using antiserum PBL-49, recognizes both sGnRH-like and cGnRH-II-like molecules in various parts of the goldfish brain. cGnRH-II (synthesized by R. Millar) showed 33% crossreactivity in this RIA with

synthetic sGnRH (Peninsula Laboratories, San Carolos, CA) as standards. Total protein content of each brain area was determined by Bradford's method (Bio-Rad).

Statistical analysis.

The data were analyzed using the Student's t test or analysis of variance (one-way or two-way) followed by the Student-Newman-Keuls test after logarithmic transformation.

4.3 RESULTS

Effects of pimozide and apomorphine on levels of brain GnRH and serum GtH in normal male fish.

In Experiment 1, pimozide at dosages of 10 and 20 nmol/g BWt increased GnRH levels in olfactory bulbs (not significant with 10 nmol/g pimozide), telencephalon and pituitary at 24 hr after treatment (Fig. 4.1). Serum GtH levels showed dose-dependent increases with treatments of 0.1 to 20 nmol/g BWt pimozide (Fig. 4.1). Figure 4.2 shows that pimozide (10 nmol/g BWt) significantly increased serum GtH levels and GnRH levels in the telencephalon and pituitary at 9 and 15 hr after treatment. Pimozide increased GnRH levels in olfactory bulbs only at 15 hr following treatment (Fig. 4.2). GnRH levels in the hypothalamus of either the vehicle or pimozide-treated fish did not show significant changes with time (data not shown). As shown in Figure 4.3, the effects of pimozide on brain GnRH levels were abolished by the concomitant administration of 10 nmol/g BWt apomorphine. The effects of pimozide on serum GtH levels were significantly reduced, but not entirely abolished by apomorphine.

Effects of pimozide and apomorphine on levels of brain GnRH and serum GtH in male fish during spawning behavior.

In the control fish (males exposed to other males), apomorphine, by itself, did not cause significant changes in the GnRH concentrations in the pituitary or in the discrete brain areas studied. Apomorphine alone caused a small decrease in serum GtH levels 24 hours after injection in Experiment 3 (Fig. 4.3), but had no such effects 4 to 10 hours after treatment (Experiment 4, Fig. 4.4). However, in males exposed to PG-females, the increases in serum GtH levels, and in GnRH concentrations in olfactory bulbs, telencephalon and hypothalamus observed at 2 hr, and in some cases at 6 hr, were abolished by apomorphine pretreatment.

As in Experiments 1, 2, 3 and 5, pimozide pretreatment alone caused significant increases in serum GtH levels, and in GnRH concentrations in the olfactory bulbs and telencephalon (Figs. 4.1, 4.2, 4.3 and 4.5). Figure 4.5 shows that the serum GtH levels in pimozide-treated fish exposed to PG-females for 2 hours increased significantly compared to pre-exposure levels and the levels in control groups (vehicle-treated fish exposed to PG-females). In contrast to the vehicle-treated fish exposed to PG-females, the pimozide treated fish exposed to PG-females had significant decreases in GnRH concentrations in the olfactory bulbs and telencephalon at both 2 and 6 hrs compared to pre-exposure levels; GnRH levels in the pituitary of pimozide-treated fish exposed to PG-females for 2 hours were significantly decreased compared to pre-exposure levels, and levels in the control group at the same sampling time. (Fig. 4.5). Serum GtH levels in pimozide-treated fish exposed to PG-females for 6 hours remained significantly higher than those in vehicle-treated fish, but not significantly different from the pre-exposure levels.

4.4 DISCUSSION

Effects of pimozide and apomorphine on levels of brain GnRH and serum GtH in normal male fish.

A major finding of the present study is that concentrations of immunoreactive GnRH in discrete brain areas and the pituitary of male goldfish are altered by treatment with the dopaminergic receptor antagonist pimozide. This, together with the studies showing direct effects of dopamine to inhibit GnRH-stimulated GtH release at the level of the pituitary in goldfish (Chang *et al.*, 1984, 1989), suggest that dopamine can act at the level of both brain GnRH neurons and pituitary gonadotrophs to influence GtH secretion. An interaction of dopaminergic pathways with the GnRH peptidergic system is also suggested by the anatomical proximity of the two neuronal systems. In goldfish, cell bodies immunoreactive for GnRH are distributed in the olfactory bulbs, the ventral telencephalon, and the preoptic region; GnRH cell bodies are most abundant in the preoptic region (Kah *et al.*, 1986). All these brain areas are rich in dopaminergic networks, and the preoptic-hypophyseal dopaminergic neurons are located close to the GnRH peptidergic neurons in the preoptic region (Kah *et al.*, 1986, 1987). Also, both dopaminergic and GnRH immunoreactive nerve fibers directly innervate pituitary gonadotrophs in the goldfish (Kah *et al.*, 1987). However, the existence in teleosts of synaptic or axoaxonal contact between dopamine and GnRH containing neurons as reported in mammals (see Silverman, 1988) remains to be demonstrated.

The stimulatory effects of pimozide on the accumulation of GnRH in discrete brain areas of goldfish can be reversed by apomorphine, indicating that the action of pimozide involves stimulation of specific apomorphine-sensitive dopaminergic receptors, but is not related to the ability of pimozide to bind to calmodulin (Weiss and Wallace, 1980). Apomorphine alone had no significant effects on brain GnRH levels when given in doses

sufficient to abolish the stimulatory effects of pimozide. These observations suggest that brain GnRH neuronal activity (in terms of the brain GnRH content) is under a constant dopaminergic inhibitory tone. The accumulation of GnRH in various brain areas following pimozide treatment may presumably result from some combination of a decrease in degradation, transport or release, and increase in synthesis of the peptide. A stimulation of GnRH synthesis following treatment with pimozide is supported by the observation that increases in GnRH levels occur in brain areas containing GnRH cell bodies (olfactory bulbs, telencephalon; Kah *et al.*, 1986). Although the present study does not address the underlying mechanisms involved, the finding that dopamine inhibits GnRH release from the goldfish preoptic-anterior hypothalamic slices and pituitary fragments *in vitro* (in Chapter 6 and 7) and that dopamine stimulates degradation of GnRH in rat synaptosomes (Marcano de Cotte *et al.*, 1980) suggest that pimozide may stimulate the synthesis and/or decrease in degradation more than it stimulates the rate of transport or release, of the GnRH peptides in the goldfish brain.

Effects of apomorphine and pimozide on levels of brain GnRH and serum GtH in male fish during spawning behavior.

Daily variations in the GtH response of male goldfish to the stimulus of PG-females have been reported (Dulka *et al.*, 1987); serum GtH levels in male goldfish show a marked elevation in response to PG-females during scotophase, but not during the middle of the photophase. In the present study, exposure of male goldfish to PG-females during the scotophase induced increases in the GnRH content in discrete brain areas and increases in serum GtH levels similar to those described previously when the exposure to PG-females was at the beginning of the photophase (Chapter 3). This study found that the increase in serum GtH levels and the increase in brain GnRH levels in males exposed to PG-females is suppressed by pretreatment with apomorphine; however, apomorphine pretreatment had no effects on brain GnRH levels and caused only a small decrease in serum levels of GtH in

males under control conditions (exposed to other normal males in Experiment 4). Notably, the apomorphine treated males showed apparently normal spawning behavior responses to PG-females (Yu and Peter, unpublished. results). These observations together suggest that apomorphine blocks the changes in brain GnRH and serum GtH levels in response to exposure to PG-females by suppressing the influence of spawning behavior with PG-females and not by evoking a nonspecific decrease in GnRH neuronal activities. Whether this entails actions of apomorphine at the brain and/or pituitary levels is not clear. It is known that apomorphine and dopamine can cause a decrease in GtH release in response to exogenous GnRH administration *in vivo* (Chang and Peter, 1983; Chang *et al.*, 1983), as well as block the effects of GnRH on dispersed pituitary cells *in vitro* (Chang *et al.*, 1984, 1989), indicating that action at the level of the pituitary gonadotrophs is likely. However, this does not explain the effects of apomorphine on brain GnRH levels in males exposed to PG-females; the results support the idea that dopamine also has a central inhibitory effect on the brain GnRH neuronal system.

To further pursue the physiological significance of the central dopaminergic system in the regulation of brain GnRH levels, we investigated the response of pimozide-pretreated male goldfish to PG-females. A most striking observation was that the pimozide-sensitive dopaminergic mechanism is not only involved in the regulation of the spontaneous secretion of GtH, but that it also influences the release of GtH in response to spawning stimuli. The results described in this study indicate that pimozide exaggerates the serum GtH response to sexual stimuli. Moreover, in concert with the large increases in serum GtH levels in the pimozide pretreated males exposed to PG-females, there was a marked reduction in GnRH levels in the olfactory bulbs and telencephalon. These observations suggest that, by blocking the inhibitory dopaminergic influences on the GnRH neurons with pimozide, a greater accumulation of GnRH occurred, and under the stimulatory influence from PG-females during spawning behavior, a greater release of GnRH

occurred.

Although the results suggest that pimozide potentiates the GtH response of males to spawning stimuli with PG-females by competitively antagonizing the inhibitory effects of endogeneous dopamine on the GnRH neuronal system, as well as blocking the inhibitory effects of dopamine on the gonadotrophs (Chang and Peter, 1983), responsiveness of the gonadotrophs to GnRH was likely also increased. Recent studies in goldfish (de Leeuw *et al.*, 1989) and in African catfish (de Leeuw *et al.*, 1988) have shown that blockade of dopaminergic receptors causes increases in pituitary GnRH receptor binding capacity. It is therefore probable that increases in serum GtH levels in pimozide-treated fish in response to spawning stimuli may also be due to an enhanced GtH output from the sensitized gonadotrophs.

The existence of a negative correlation between brain GnRH content and the surge in serum GtH levels in ovulating female goldfish has been suggested as evidence for involvement of GnRH in stimulating GtH secretion (Chapter 2). Similar data showing an inverse relationship between brain GnRH and plasma GtH levels have been reported for both male and female roach around the spawning period (Breton *et al.*, 1988a,b). On the contrary, in male goldfish undergoing spawning behavior with PG-treated females, such a negative correlation was not observed (Chapter 3, and present study). However, a negative correlation between brain GnRH content and serum GtH levels was observed in male fish pretreated with pimozide undergoing spawning behavior with PG-treated females. Notably, pimozide pretreated males had increased brain levels of GnRH, and during spawning behavior such males had a surge in serum GtH levels much greater than those of vehicle-treated males undergoing spawning behavior with PG-females. Moreover, the preovulatory surge in serum GtH levels in females (in Chapters 2, 5) and the surge in serum GtH levels in pimozide pretreated males undergoing spawning behavior are of similar magnitudes. In both cases, the release of GtH is presumably driven, at least in part,

by a surge in release of GnRH, leading to a short-term reduction in brain GnRH levels.

In conclusion, the presence of a tonic inhibitory action of dopamine on both the brain GnRH neuronal system and pituitary gonadotrophs provides an explanation for the observed changes in brain GnRH and serum GtH levels during spawning behavior with PG-treated females in normal and pimozide-treated goldfish. Treatment with pimozide blocked the central dopaminergic inhibition on the GnRH neurons, resulting in a larger pool of GnRH available for the activation of pituitary GtH secretion upon receiving the pheromonal signal from PG-females during spawning behavior. The simultaneous activation of pituitary GtH secretion by both augmented release of GnRH peptide and removal of the inhibitory effects of dopamine directly on gonadotrophs resulted in the observed large increase in serum GtH levels in the pimozide-treated male goldfish during spawning behavior. On the other hand, the amount of GnRH released to the pituitary during spawning behavior in normal male goldfish was relatively less because of the central inhibitory effects of dopamine on the GnRH neuronal system, and the GnRH released induced only a small increase in GtH secretion in the presence of the inhibitory effects of dopamine on pituitary gonadotrophs.

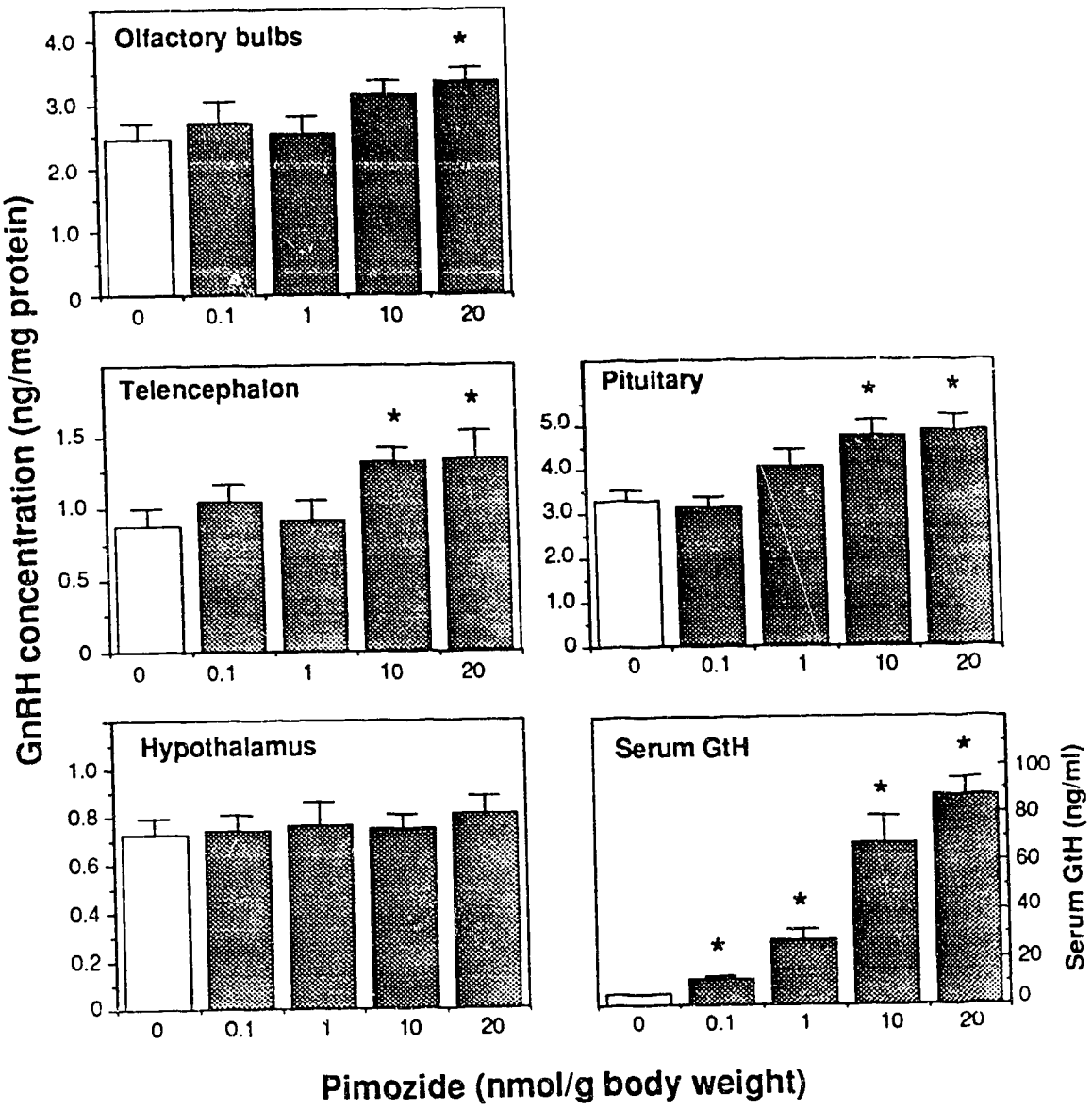

Fig. 4.1. Effects of different doses of pimozide on serum GtH levels, and GnRH concentrations in discrete forebrain areas and pituitary of male goldfish, at 24 hr after injection. Each column represents mean ± SEM (n=8-10). * indicates a significant difference ($p < 0.05$, one-way ANOVA followed by Student-Newman-Keuls test) from the respective control (vehicle injected) group.

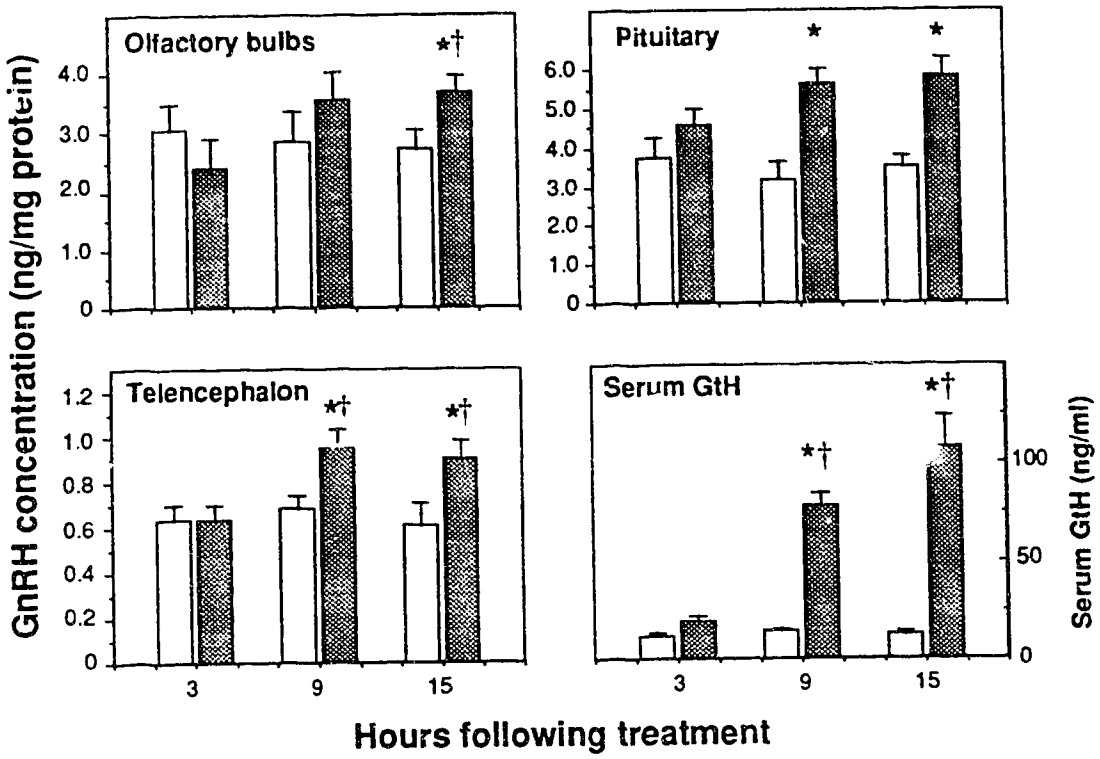

Fig. 4.2. Time-course of effects of pimozide (10 nmol/g BWt) on serum GtH levels, and GnRH concentrations in olfactory bulbs, telencephalon and pituitary of male goldfish. Each column represents mean ± SEM (n=8). * and † indicate values significantly different from the corresponding control (vehicle-injected; open bars) values and 3 hr (pimozide-injected; shaded bars) values, respectively ($p < 0.05$, Student-Newman-Keuls test after two-way ANOVA).

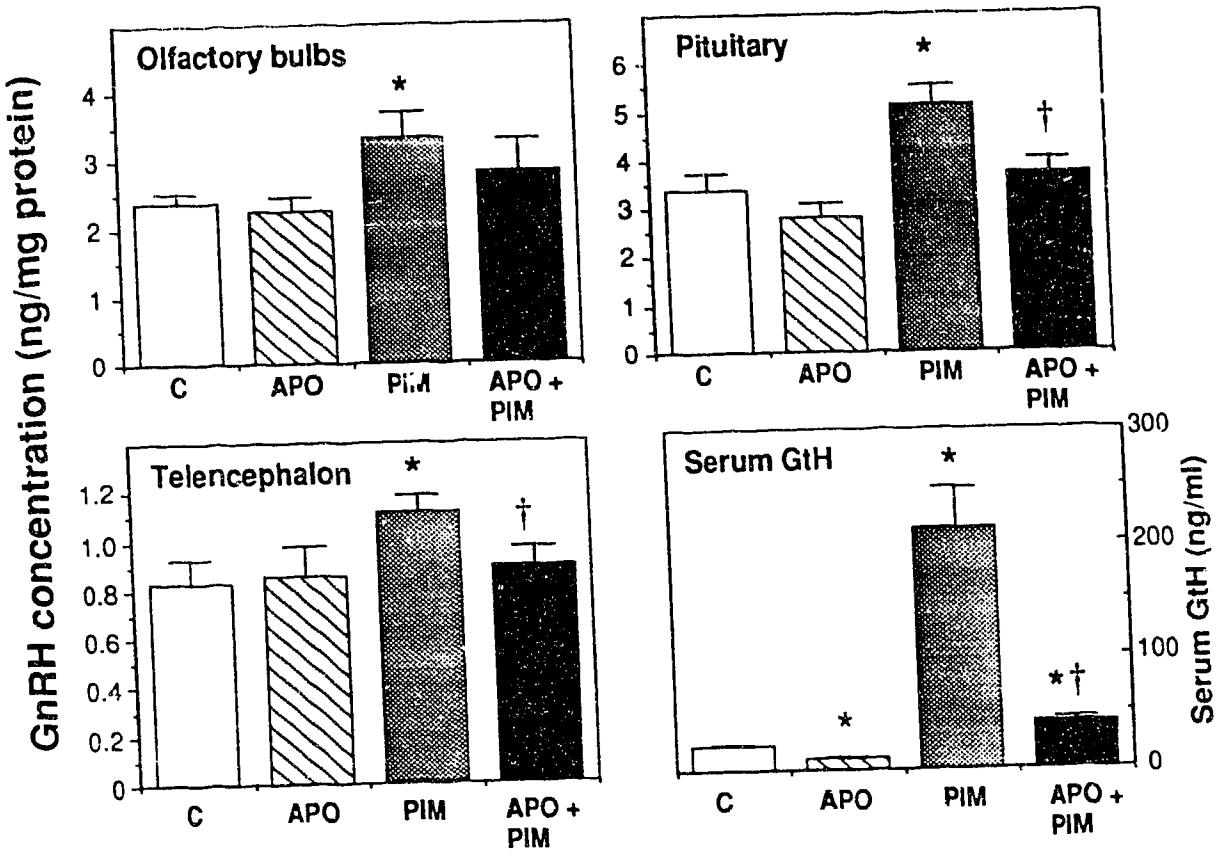

Fig. 4.3. Effects of pimozide (PIM; 10 nmol/g BWt), apomorphine (APO; 10 nmol/g BWt), or a combination of pimozide and apomorphine on serum GtH levels, and GnRH concentrations in olfactory bulbs, telencephalon and pituitary of male goldfish at 24 hr after injection. Each column represents mean ± SEM (n=9-10). * and † indicate values significantly different from the respective control (C; vehicle injected) group and the PIM group values, respectively ($p < 0.05$, one-way ANOVA followed by Student-Newman-Keuls test).

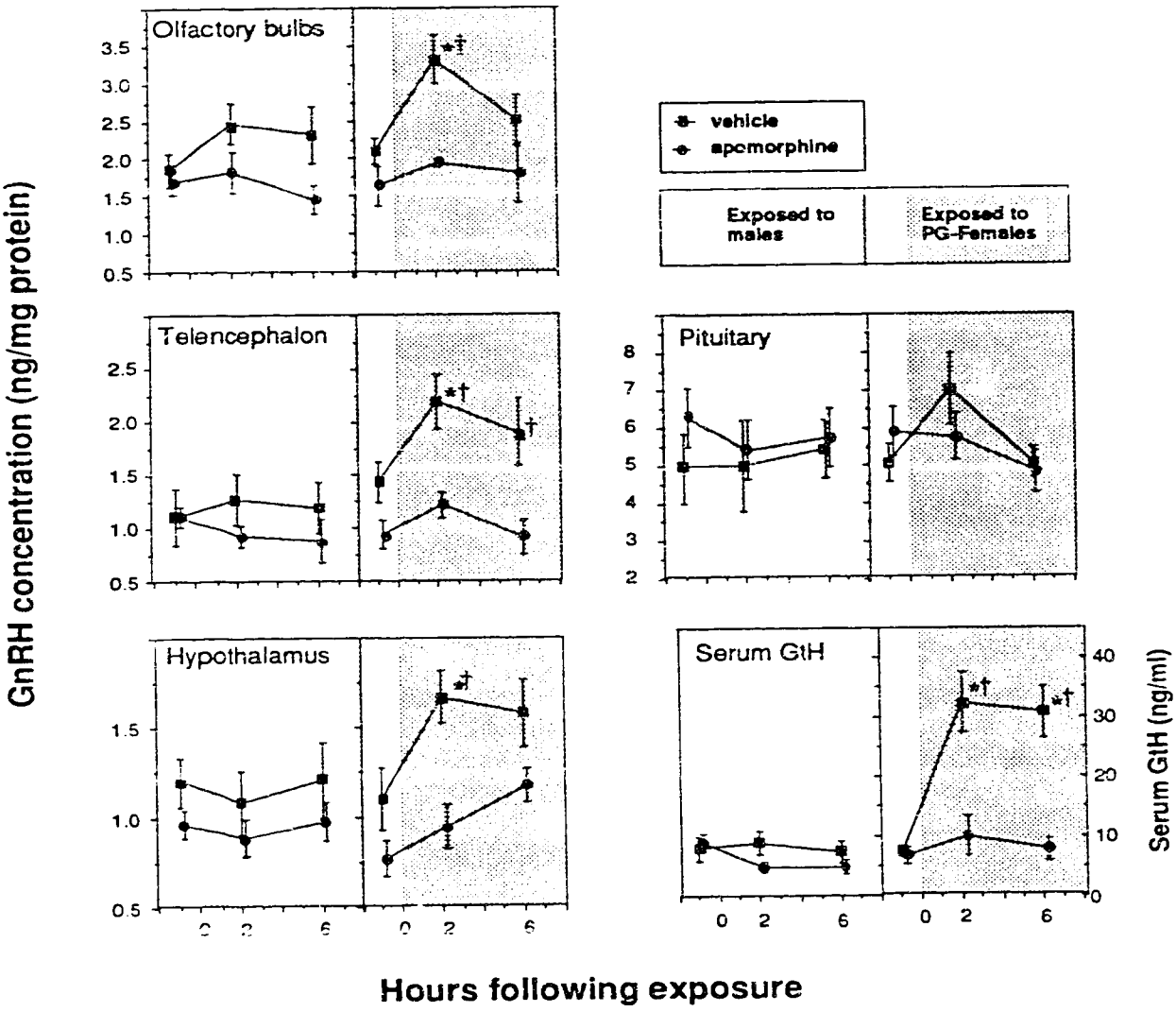

Fig. 4.4. Temporal changes in serum GtH levels, and GnRH concentrations in discrete forebrain areas and pituitary of male goldfish pretreated with either vehicle or apomorphine (33 nmol/g BWt) at 5 hr before the onset of exposure to either normal males or PG-females. Values represent mean ± SEM (n=5-10). Exposure to either normal males or PG-females began at 2 hr after the beginning of the dark phase and fish were sampled at -1, 2, 6 hour relative to the onset of exposure. * and † indicate values significantly different from the corresponding pre-exposure (-1 hr) values and control (vehicle-injected) values, respectively (Student-Newman-Keuls test after two-way ANOVA).

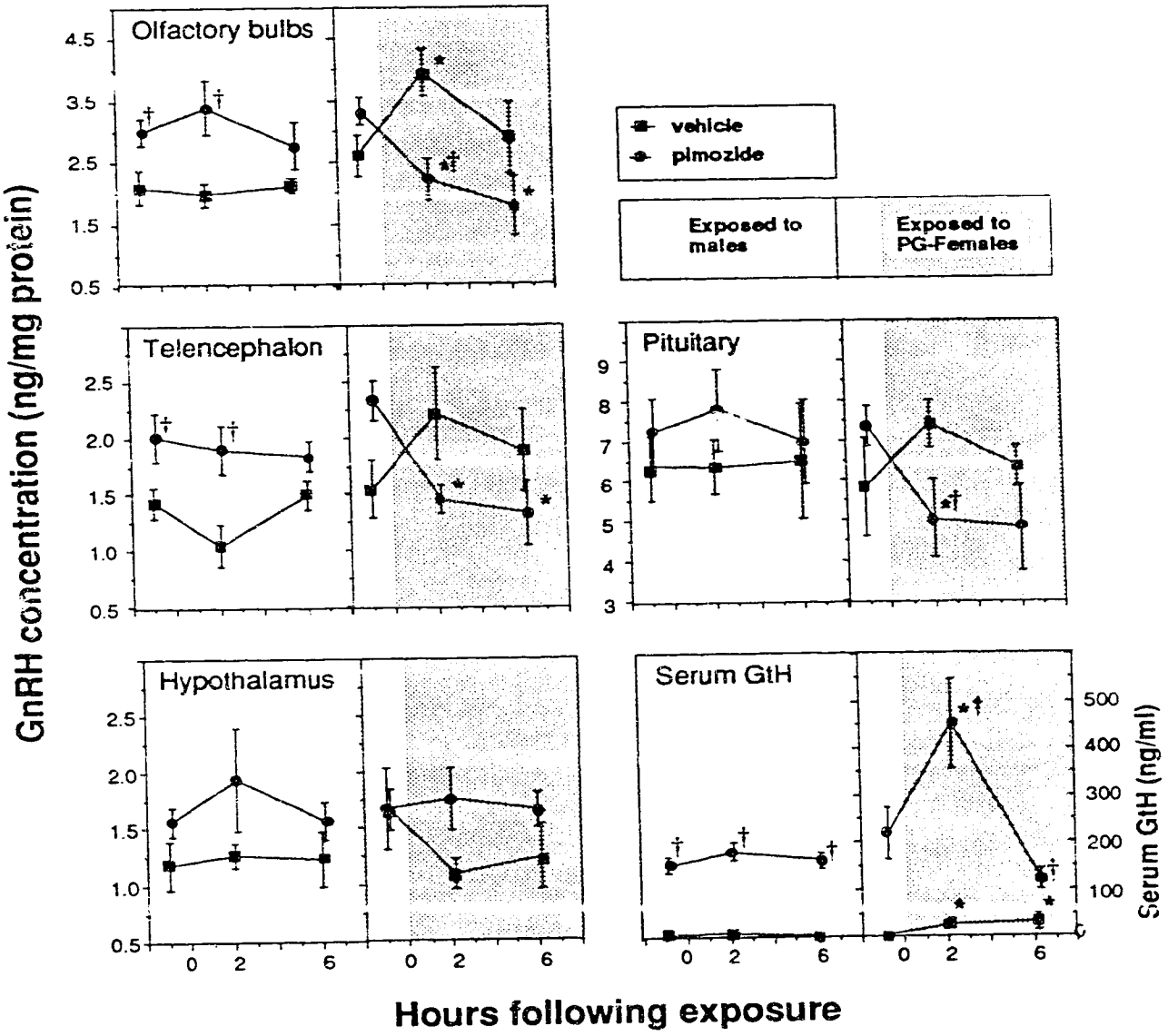

Fig. 4.5. Temporal changes in serum GtH levels, and GnRH concentrations in discrete forebrain areas and pituitary of male goldfish pretreated with either vehicle or pimozide (10 nmol/g BWt) at 12 hr before the onset of exposure to either normal males or PG-females. Values represent mean ± SEM (n=6-10). Exposure to either normal males or PG-females began at 2 hr after the beginning of the dark phase and fish were sampled at -1, 2, 6 hour relative to the onset of exposure. * and † indicate values significantly different from the corresponding pre-exposure (-1 hr) values and control (vehicle-injected) values, respectively (Student-Newman-Keuls test after two-way ANOVA).

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5. CHANGES IN BRAIN LEVELS OF GONADOTROPIN-RELEASING HORMONE AND SERUM LEVELS OF GONADOTROPIN AND GROWTH HORMONE IN MALE GOLDFISH DURING SPAWNING[1]

5.1 INTRODUCTION

The ovulatory surges of gonadotropin (GtH; Stacey *et al.*, 1979) and growth hormone (GH; Marchant, 1983) in female goldfish have been described previously . A surge in serum GtH levels also occurs in male goldfish held together with females undergoing an ovulatory surge of GtH release (Kobayashi *et al.*, 1986; Stacey *et al.*, 1989). Recent studies showed that gonadotropin-releasing hormone (GnRH) is capable of stimulating both GtH and GH release in goldfish (Marchant and Peter, 1989; Marchant *et al.*, 1989), providing the possibility that GnRH may be involved in the ovulatory surge of both GtH and GH. Significant decreases in radioimmunoassayable total GnRH concentrations in discrete brain areas in association with the ovulatory surge of GtH occur in female goldfish (Chapter 2). However, the relationship between the spawning induced GtH surge and changes in brain levels of GnRH in male goldfish have not been examined. In the present study, the temporal changes in brain GnRH levels, and serum GtH and GH levels in male goldfish exposed to ovulatory females were studied. In addition, similar hormone measurements were made in ovulating female goldfish to enable comparison with the timing of hormone changes in males and to confirm previous findings.

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[1] A version of this chapter has been submitted for publication: Yu, K.L., Peng, C., and Peter, R.E. Can. J. Zool.

5.2 MATERIALS AND METHODS

Experimental animals.

Sexually mature male and female goldfish were held separately in an 1800-liter flow-through aquarium at 12-13°C on a simulated natural (Edmonton) photoperiod from March to May until experiments were conducted. Two weeks before experiments, eighty-one males having expressible milt (upon lightly pressing the abdomen) were selected and acclimated in a 225-liter flow-through aquarium at 15°C. Ninety females with a distended and soft abdomen were selected and equally divided among six 96-liter flow-through aquaria (fifteen fish in each aquarium) at 12°C. All fish were acclimated to 16 hours light and 8 hours darkness starting two weeks before experiments. Three days before experiments, male goldfish were warmed to 20°C. In three aquaria containing females, designated as spawning aquaria, induction of spontaneous ovulation was attempted by raising the water temperature from 12°C to 20°C over a period of 10 hours (from 1600 hr Day 1 to 0200 hr Day 2) and the addition into each aquarium of artificial floating vegetation to serve as a spawning substrate (1600 hr Day 1). Spawning behavior in female and male goldfish naturally occurs at the time of ovulation if a spawning substrate is present. At 0800 hr on Day 2, twelve sexually mature males were transferred into each of the three spawning aquaria, and 36 males were transferred into another 225-liter male control aquarium (with floating vegetation but no females) at 20°C. In the remaining three aquaria containing females, designated as control aquaria, temperature was increased similarly, but artificial floating vegetation and males were not introduced.

Blood and brains were sampled from both female and male fish taken randomly from the aquaria at various times from 1300 hour on Day 1 to 1300 hour on Day 3 at times indicated in Figs. 5.1-5.3. Male fish (n=9) sampled at 1300 hr on Day 1, three days after warming to 20°C but prior to the transfer to the male control and spawning aquaria,

represent the pre-exposure group of male fish. Pre-exposure female fish (n=9) were sampled randomly from the aquaria (female control and spawning) at 1300 hr on Day 1, prior to the addition of artificial floating vegetation and increase in water temperature (started at 1600 hr Day 1) and their exposure to the sexually mature males (introduced at 0800 hr Day 2). At 1300 hour on Day 2, female goldfish (n=18) sampled from the spawning aquaria were divided into two groups based on their serum GtH levels; individual fish in which serum GtH levels were significantly higher (Student's t-test for single samples) than the mean GtH levels of the female control fish were considered to be in the initial stages of the ovulatory surge of GtH and were designated as "ovulatory"; fish in which serum GtH levels did not differ significantly from the control fish were designated as "nonovulatory". At 0600 hr and 0800 hr on Day 3, ovulation was confirmed by lightly squeezing the abdomen of fish to eject a stream of oocytes from the ovipore.

For blood and brain sampling, fish were anesthetized in 0.05% tricaine methanesulfonate. Blood was sampled from the caudal vasculature, and collected serum was frozen on dry ice and stored at -30°C until radioimmunoassay (RIA) for GtH. The brain and pituitary were rapidly removed after blood sampling, and different brain parts (olfactory bulbs, telencephalon, hypothalamus) were rapidly dissected on a glass petri dish over ice. The pituitary and each brain area were immersed in 2 ml of ice-cold 2 M acetic acid, and GnRH extracted by sonication and centrifugation as described previously in Chapter 2 (section 2.2). Lyophilized extracts were reconstituted in RIA assay buffer and assayed for total GnRH immunoactivity.

The experiment was conducted twice and data from the two experiments were combined for statistical analysis.

Radioimmunoassays for GtH and GnRH

Serum GtH levels were determined by a RIA for common carp maturational GtH as

described previously (Peter *et al.*, 1984). Total GnRH immunoactivity in each brain area was measured using a double antibody radioimmunoassay (RIA) with ^{125}I-[Trp^7, Leu^8]-GnRH (^{125}I-sGnRH) as tracer and synthetic sGnRH (Peninsula Laboratories, San Carlos, CA) as standards (see Sections 2.2, 3.2). The brain and pituitary of goldfish contains sGnRH and [His^5, Trp^7, Tyr^8]-GnRH (cGnRH-II)-like molecules (see Chapter 8), and, as reported previously, the RIA using antiserum PBL-49, recognizes both sGnRH and, to a lesser extent cGnRH-II (33% crossreactivity compared to synthetic sGnRH standards). Total protein content of the extracts was determined by Bradford's method (Bio-Rad).

Statistical analysis

The data were log-transformed and analyzed using one-way ANOVA followed by the Duncan's multiple range test to compare changes over time within each of the experimental groups and to compare data from all experimental groups at each sampling time.

5.3 RESULTS

Hormones in female goldfish.

GtH. The periovulatory changes in GtH are shown in Fig. 5.1. At 1300 hr on Day 2, control fish had serum GtH levels similar to those of the pre-exposure group (at 1300 on Day 1). Twelve female fish (33 % of fish sampled) from the spawning aquaria sampled at 1300 hr on Day 2 had significantly higher serum GtH levels compared with the respective control fish and were designated as ovulatory fish (expected to undergo the ovulatory GtH surge); twenty-four fish had serum GtH levels similar to those of the control fish and were designated as nonovulatory. Serum GtH levels of nonovulatory females sampled at 0600 hr and 1300 hr on Day 3 were not significantly different from the

respective control or pre-exposure values. From 0600 hr to 0800 hr on Day 3, twenty-two (39%) out of fifty-six females in the spawning aquaria were found to be ovulated; ovulated fish (n=12) sampled at 0600 hr on Day 3 were found to have high serum GtH levels, indicative of the ovulatory surge of GtH. Serum GtH levels of the ovulated females (n=10) sampled at 1300 hr on Day 3 were reduced compared to the levels at 0600 and Day 3, but were still significantly higher than those in the nonovulatory and control fish.

GH. In the nonovulatory and control fish, serum GH levels remained unchanged with time during the experimental period. Serum GH levels in the ovulatory fish were significantly increased compared to nonovulatory and control fish at 1300 hr on Day 2, and 0600 hr and 1300 hr on Day 3 (Fig. 5.1).

GnRH. The periovulatory changes of GnRH concentration in discrete brain areas and the pituitary are shown in Figs. 5.2, 5.3. Briefly, GnRH concentrations in the olfactory bulbs, telencephalon, hypothalamus and pituitary of the ovulatory fish were significantly lower than those in the nonovulatory and control fish at 1300 hr on Day 2, approximately 17 hr before ovulation. At 0600 hr on Day 3, when the serum GtH levels were highest, and ovulation had already occurred, the GnRH concentrations in the telencephalon, hypothalamus and pituitary remained significantly lower than those in the nonovulatory and in the control fish; GnRH concentrations in the olfactory bulbs were significantly different from those in the control fish, but not from those in the nonovulatory fish (Fig. 5.2). GnRH concentrations in discrete brain areas and the pituitary of the nonovulatory fish did not differ significantly from those of the control females at any sampling time. At 1300 hr on Day 3, there were no significant differences among ovulatory, nonovulatory and control fish in the GnRH concentrations in various brain areas and the pituitary. GnRH concentrations in discrete brain areas and the pituitary of the control fish did not significantly change with time during the experimental period. There were, however general decreases in GnRH concentrations in the olfactory bulbs, telencephalon and hypothalamus in nonovulatory fish during the experimental period (Fig

5.2, 5.3). GnRH concentrations in the olfactory bulbs of the nonovulatory fish showed gradual decreases from 1300 hr on Day 1 to 0600 hr on Day 2 and were significantly lower than the pre-exposure values at 1300 hr on Day 3 (Fig. 5.2); GnRH concentrations in the telencephalon and hypothalamus were lower at 1300 hr on Day 2 and at 0600 hr on Day 3 compared to the pre-exposure values (Figs. 5.2, 5.3); GnRH concentrations in the pituitary of the nonovulatory fish did not significantly change with time during the experimental period (Fig. 5.3).

Hormones in male goldfish

GtH. In two spawning aquaria, all female goldfish failed to ovulate and consequently no spawning occurred in these males (nonspawning males). The serum GtH levels of the nonspawning males (n=6) were similar to those of the control males at all sampling times (Fig. 5.1). In control fish, serum GtH levels did not change with time except for an increase at 0600 hr on Day 3 compared to pre-exposure levels. In nonspawning males (with nonovulatory fish), serum GtH levels showed small, but significant increases with time until 0600 hr on Day 3 over the experimental period (Fig. 5.1). Serum GtH levels of spawning males showed increases over control males at 1300 hr on Day 2 (17 hr prior to ovulation), reached highest values at the time of ovulation (0600 hr on Day 3) and declined to values above both the nonspawning and control levels at 1300 hr on Day 3.

GH. Serum GH levels of the male fish in spawning aquaria were not significantly different from those of the male fish in the control aquaria at all sampling times except at 0600 hr on Day 3 (Fig. 5.1). The serum GH levels of the nonspawning males were similar to those of the control males at all sampling times (Fig. 5.1). In both nonovulatory and control fish, serum GH levels remained unchanged with time during the experimental period.

GnRH. The temporal changes in GnRH concentrations in discrete brain areas and the pituitary of male goldfish during spawning with ovulatory females are shown in Fig. 5.2, 5.3. GnRH concentrations in discrete brain areas and the pituitary of the control and nonspawning fish did not change significantly with time during the experimental period. GnRH concentrations in the olfactory bulbs and telencephalon of the spawning male fish did not differ from the pre-exposure values except at 0600 hr on Day 3. GnRH concentrations in the hypothalamus of the spawning males at 2200 hr on Day 2 and 0600 hr on Day 3 were significantly different from the pre-exposure values (Fig. 5.2). GnRH concentrations in the pituitary of the spawning males showed an increase at 1300 hr on Day 2 and a decrease at 0600 hr on Day 3 compared to the pre-exposure values (Fig. 5.1). At 0600 hr on Day 3, GnRH concentrations in the olfactory bulbs, telencephalon hypothalamus and pituitary of spawning males were significantly lower than those of the nonspawning and control males. GnRH concentrations in the hypothalamus and pituitary of the spawning males were significant lower than those in the control fish at 2200 hr on Day 2 (Fig. 5.3).

5.4 DISCUSSION

This study demonstrates that a marked increase in serum GtH and GH levels occurs only in male goldfish exposed to spontaneously ovulating females, but not in control or nonspawning males. These results suggest that the changes in hormone levels in spawning male goldfish are mainly associated with the exposure to ovulating females but not to non-specific stimuli from nonovulatory females, artificial floating vegetation or other males. Small increases in plasma GtH levels during the scotophase in control male goldfish , and in males exposed to nonovulatory females have been reported previously (Kobayashi *et al.*, 1986); similar small increases with time in serum GtH levels of the nonspawning

males were observed in this study.

The demonstration of a GtH surge in male goldfish during spawning with spontaneously ovulating females in the present study confirms earlier studies (Kobayashi *et al.*, 1986; Stacey *et al.*, 1989). In a series of studies (Stacey and Sorensen, 1986; Dulka *et al.*, 1987; Sorensen *et al.*, 1988; Stacey *et al.*, 1989), it was demonstrated that the increase in serum GtH levels during spawning in male goldfish could be accounted for by two pheromones released in temporal sequence from ovulatory females: a preovulatory primer pheromone, 17α20β-dihydroxy-4-pregnen-3-one (17,20β-P) and a postovulatory releaser pheromone, prostaglandin (PG). According to this hypothesis, males exposed to high concentrations of 17,20β-P, or other as yet unknown pheromones from preovulatory females have an increase in serum GtH levels as early as 15 hours prior to ovulation (Stacey *et al.*, 1989). At the time of ovulation, PG-pheromones released from the ovulating females stimulate spawning behavior in males and increase serum GtH levels as a result of behavioral interaction. In the present study, male goldfish exposed to ovulatory females had serum GtH levels significantly higher than those in the control fish at approximately 17 hours prior to ovulation. At 2200 hr on Day 2, approximately 8 hr prior to ovulation, serum GtH levels in spawning male goldfish had increased sharply, and reached the apparent peak of the surge at the time of ovulation by the accompanying females (at 0600 hr on Day 3). This observation correlates well with the 17,20β-P released into the water by ovulating females, which is detectable at about 8 hr prior to ovulation and reaches peak levels at the time of ovulation (Stacey *et al.*, 1989).

In the spawning male goldfish, in association with the increases in serum GtH and GH levels, there were marked reductions of GnRH concentrations in the olfactory bulbs, telencephalon, hypothalamus and pituitary. This finding is analogous to the observations in female goldfish undergoing spontaneous ovulation (in this study and Chapter 2). Observation of an inverse relationship between brain GnRH and serum GtH levels has also been reported in male and female roach during the spawning period (Breton *et al.*, 1988a,

b).

In the present study, the profile of the changes in brain and pituitary GnRH levels of spawning male goldfish showed some temporal differences with those of ovulatory females. Marked reductions in brain and pituitary GnRH levels were observed in ovulatory fish at about 17 hours prior to ovulation (in this study and Chapter 2); GnRH levels in brain and pituitary of spawning males had not changed by this time (pituitary GnRH levels showed a small increase compared to the pre-exposure levels, Fig. 5.2). About 8 hours prior to the ovulation of females in the aquaria, spawning males exhibited significant decreases in GnRH content of the hypothalamus and pituitary (compared to controls), but not in the olfactory bulbs and telencephalon. Thus, the spawning-induced reduction in brain and pituitary GnRH levels of males occurs at a time later than that in ovulatory female goldfish.

The mechanisms underlying the reduction in brain levels of GnRH in spawning male goldfish in the present study are not entirely clear. The temporal correlation between changes in brain GnRH and serum GtH levels in the present study would suggest that 17,20β-P pheromones from the preovulatory female goldfish stimulate GtH secretion in the males by acting through the brain GnRH neuronal system. However, Dulka (1989) found that exposure of male goldfish to 0.5 nM 17,20β-P for twelve hours significantly stimulated serum GtH levels but did not alter the GnRH concentrations in the telencephalon, hypothalamus and pituitary. One explanation for the inability of 17,20β-P exposure to influence brain GnRH levels is that the female preovulatory pheromone consists of more than the identified 17,20β-P. It has been suggested that other related steroids, such as 17α–hydroxyprogesterone are capable of stimulating GtH and milt increases in male goldfish (in Stacey *et al.*, 1989). This may also explain why the serum GtH levels of the spawning males exposed to preovulatory females or their waterborne pheromones increased gradually (Kobayashi *et al.*, 1986; Stacey *et al.*, 1989) whereas

serum GtH levels peak within 15 min following addition of synthetic 17,20β-P to the aquarium water (Dulka *et al.*, 1987).

This study demonstrated that serum GH increased in spontaneously ovulating goldfish, thus confirming the previous results of Marchant (1983). In addition, increases in serum GH levels were also found in male goldfish during spawning with spontaneously ovulating females. The present study is also consistent with the report by Dulka (1989) that serum levels of GH in male goldfish are increased following exposure for 8 hours to a female induced to ovulate by injection of human chorionic gonadotropin (hCG). However, it is intriguing that serum GH levels did not increase in male goldfish during exposure to either 17,20β-P or PG pheromones, or a combination of both, added to the aquarium water, although the serum GH levels were increased after 8 hours of continuous exposure to 17,20β-P in one experiment (Dulka, 1987, 1989). In view of the potentiating effects of GH on GtH-induced gonadal steroidogenesis in teleosts (Singh *et al.*, 1988; van der Kraak *et al.*, 1989), the serum GH surges observed during spawning in male and female goldfish may be important to elicit the full gonadal response to the GtH surges.

The dual control of GtH secretion by GnRH and dopamine (DA) has been well demonstrated in goldfish and other freshwater teleosts (for reviews, see Peter *et al.*, 1986; de Leeuw *et al.*, 1987). In goldfish, the two forms of GnRH identical to sGnRH and cGnRH-II have been characterized in brain, pituitary (in Chapter 7) and serum (Peter *et al.*, 1989). Recent studies in goldfish have shown that GnRH peptides are capable of stimulating both GtH and GH release *in vivo* (Marchant *et al.*, 1989; Marchant and Peter, 1989), as well as *in vitro* from pituitary fragments (Marchant *et al.*, 1989) or dispersed pituitary cells (Chang *et al.*, 1989), suggesting direct actions on both gonadotrophs and somatotrophs. In the present study, the spawning-induced increases in serum levels of GtH and GH of both male and female goldfish are associated with decreases in GnRH concentrations in the olfactory bulbs, telencephalon and pituitary. These results suggest

that GnRH may be involved in the stimulation of both GtH and GH release during spawning in both male and female goldfish. However, since serum GH levels increase at the same time as serum GtH levels following injection of ([D-Ala^6, Pro^{9}NEt]-mammalian GnRH (Marchant *et al.*, 1989), the observation in spawning male goldfish that serum GtH levels, but not serum GH levels, significantly increased prior to ovulation indicates that other neurohormones are also involved in the regulation of GH release. An alternate explanation is that the GnRH forms released during spawning differentially stimulate GtH and GH release. Differential activity of sGnRH and cGnRH-II in stimulating GtH and GH release *in vitro* from cultured goldfish pituitary cells has been suggested; while the two forms of GnRH are equipotent in stimulating GH release, cGnRH-II is more potent than sGnRH in stimulating GtH release (Chang *et al.*, 1989). Since the present study used an antiserum that crossreacts with both sGnRH and cGnRH-II, the possible differential activation of the two GnRH forms in male goldfish during spawning awaits the availability of specific RIAs for cGnRH-II and sGnRH.

The direction of changes in brain and pituitary GnRH levels in male goldfish during spawning with spontaneously ovulating females in this study is clearly different from that described in male goldfish undergoing spawning behavior with PG-treated females; male goldfish exposed to PG-treated females have small, but significant increases in serum GtH levels, and increased levels of GnRH in the olfactory bulbs, telencephalon and hypothalamus, but not the pituitary (in Chapter 3). The differential regulation of GnRH neuronal activity in male goldfish during these two conditions supports previous evidence that two separate mechanisms may be involved (Sorensen *et al.*, 1987, 1988; Dulka, 1989; Sorensen *et al.*, 1989). Nevertheless, results from the present study are consistent with the previous interpretation that exposure to PG pheromones released from PG-treated females produced a major stimulatory effect on the synthesis of brain GnRH, but not on GnRH release in the pituitary, resulting in the observed increases in brain GnRH levels and

small increases in serum GtH levels. On the other hand, male goldfish exposed to ovulating females (which presumably release both preovulatory 17,20β-P and postovulatory PG pheromones) likely have a surge release of GnRH in order to trigger the surge in GtH release, resulting in a net decrease in brain GnRH levels. In Chapter 4, it was found that male goldfish pretreated with the dopamine antagonist pimozide undergoing spawning behavior with PG-treated females also have a reduction of brain and pituitary GnRH levels in association with a large surge in serum GtH levels. These results suggest that DA receptor blockade prior to stimulation by postovulatory PG pheromones can also induce a surge in serum GtH levels and a reduction of brain GnRH levels similar to that observed in male goldfish during spawning with ovulating females. A decrease in DA inhibition of pituitary GtH secretion has been suggested to account for the rapid increase in serum GtH levels in male goldfish during exposure to the preovulatory 17,20β-P pheromones (Dulka, 1989). By measuring the ratio of dihydroxyphenylacetic acid to DA content, Dulka (1989) showed that water-borne 17,20β-P caused a rapid (15 minutes) decrease in DA turnover in the pituitary of male goldfish. Since the serum GtH surge during spawning in male goldfish can presumably result from a decrease in dopaminergic inhibition at gonadotrophs (see Peter *et al.*, 1986) and an increase in GnRH release, it would be of interest to determine whether the reduction in pituitary dopaminergic activities during exposure to water-borne 17,20β-P continues during the extended period of spawning. However, a reduction in pituitary dopaminergic activities in males during spawning is apparently not consistent with results in the present study showing an increase in serum GH hormones and results from others showing that dopamine stimulates GH secretion in goldfish (Chang *et al.*, 1985).

In summary, the present study demonstrates that male goldfish spawning with ovulatory females have a reduction in brain GnRH levels in association with increases in serum GtH and GH levels , similar to the those observed in spontaneously ovulating females. Although the precise interrelationship between pheromonal stimulation, inhibition

of pituitary dopamine turnover, activation of brain GnRH, and pituitary GtH and GH secretion during spawning in male goldfish appears to be complex, the present results suggest that activation of the GnRH neuronal system may be a common pathway for the stimulation of pituitary GtH and GH secretion in both male and female goldfish during spawning.

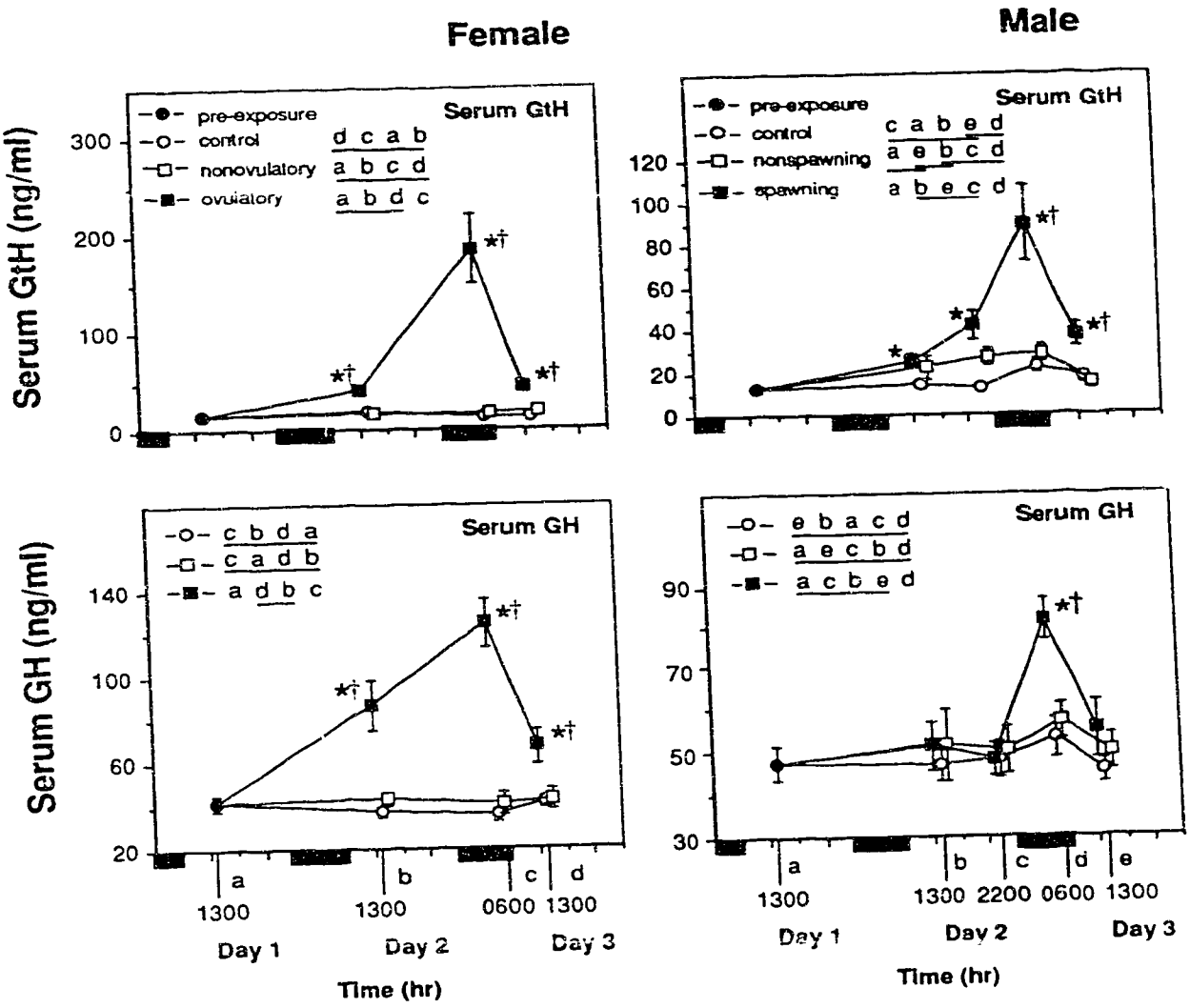

Fig. 5.1. Temporal changes in serum GtH and GH levels at various times during the periovulatory period in female goldfish, and in male goldfish during spawning with spontaneously ovulating females. Values represent mean ± SEM. The number of fish in each group: n = 16 for control female group, 16-22 for nonovulatory group, 10-12 for ovulatory group, 11-16 for control male group, 6 for nonspawning male group, and 11 for spawning male group. Fish were kept on a 16L:8D photoperiod (darkened horizontal bars represent dark period). The sampling times are indicated by lines and letters (a to d for females and a to e for male fish). Analysis of variance and Duncan's multiple range test were used to compare data from all experimental groups at each sampling time (* and † indicate values significantly different from the respective control group and nonspawning or nonovulatory group values, respectively at $p < 0.05$), and to compare changes over time within each of the experimental groups (underlined letters represent means which are statistically equivalent at $p > 0.05$).

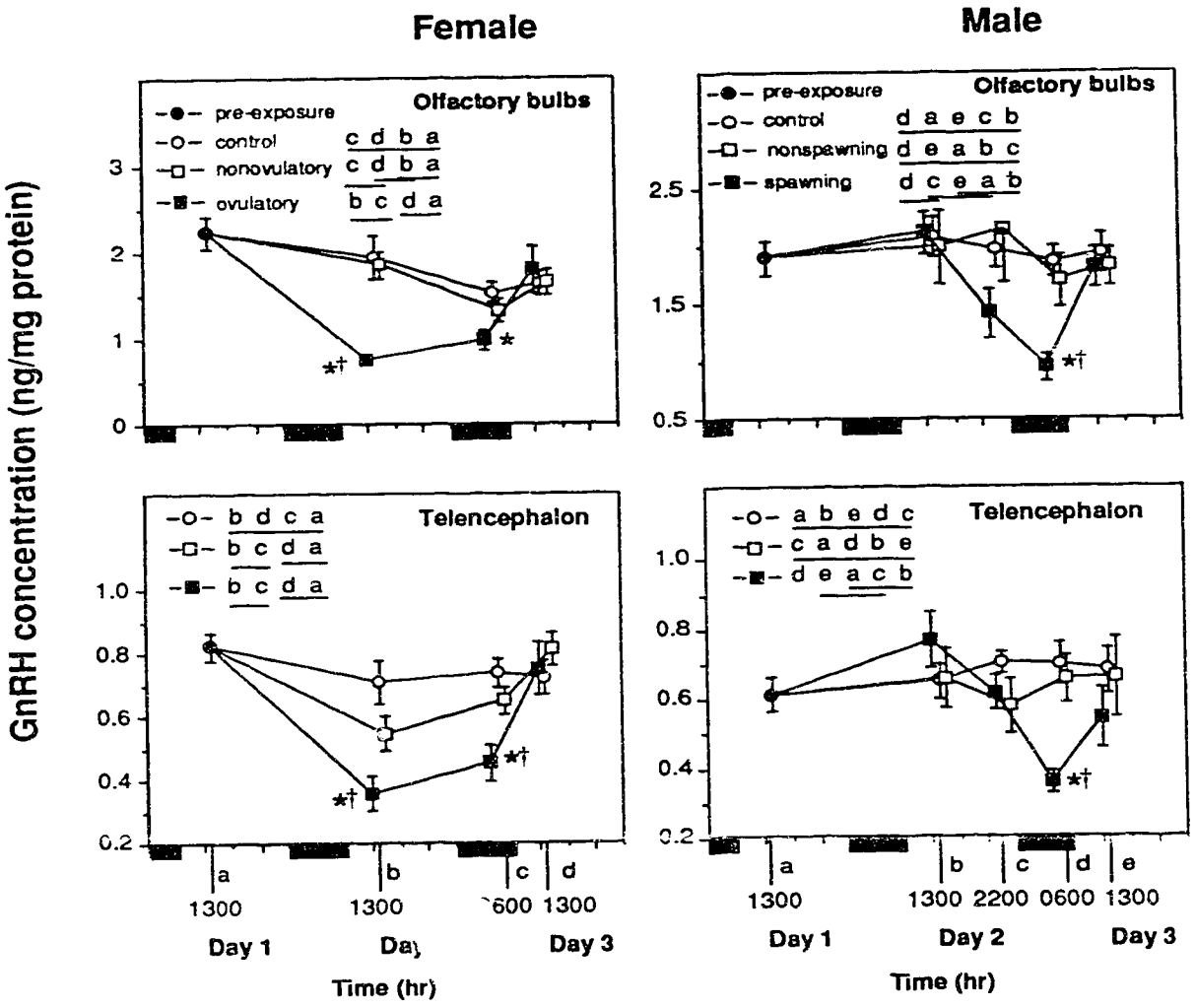

Fig. 5.2. Temporal changes in GnRH concentrations in olfactory bulbs and telencephalon at various times during periovulatory period in female goldfish, and during spawning with spontaneously ovulating females in male goldfish. Explanations and abbreviations are the same as those of Fig. 5.1.

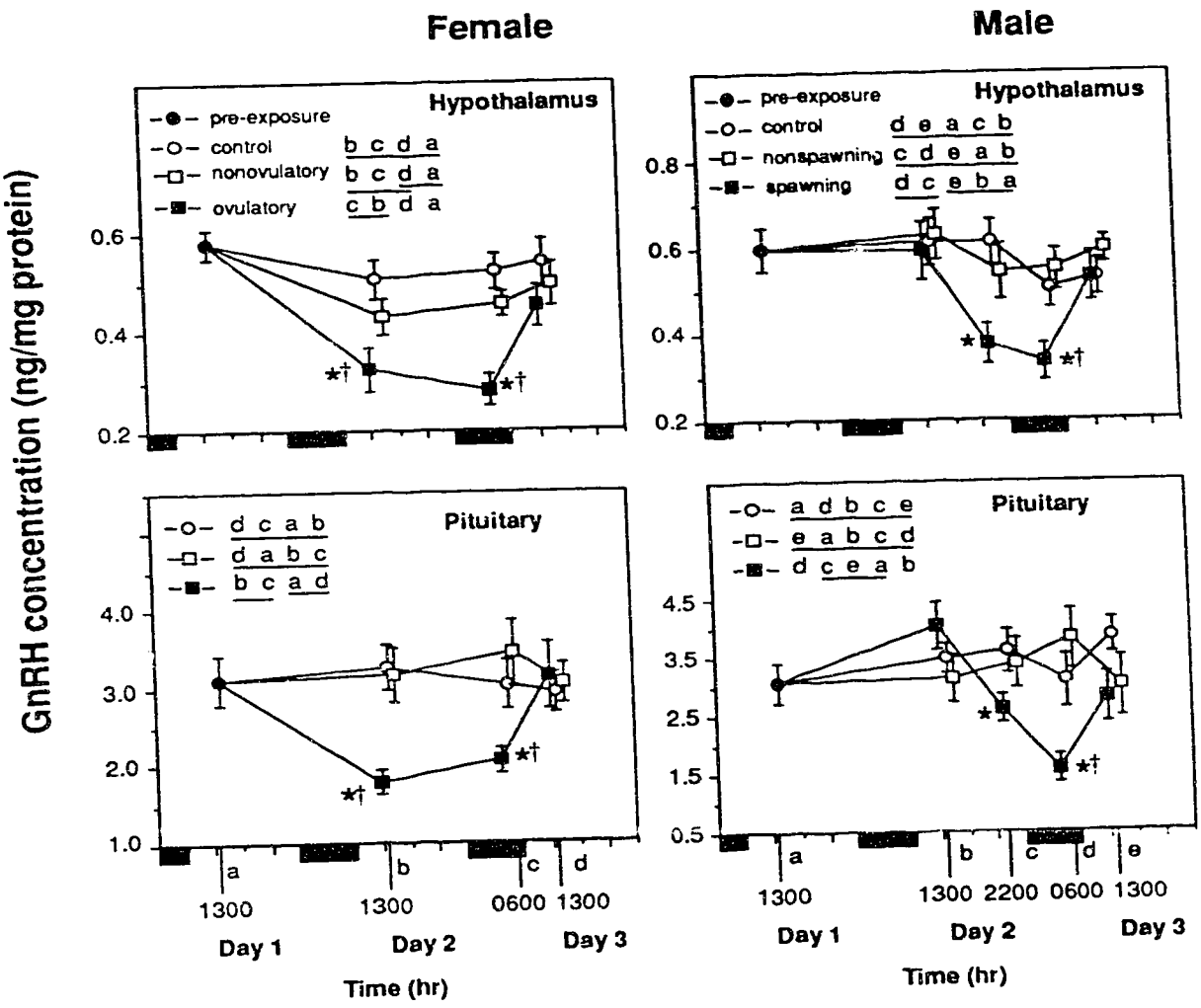

Fig. 5.3. Temporal changes in GnRH concentrations in the hypothalamus and pituitary at various times during periovulatory period in female goldfish, and during spawning with spontaneously ovulating females in male goldfish. Explanations and abbreviations are the same as those of Fig. 5.1.

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6. *IN VITRO* RELEASE OF GONADOTROPIN-RELEASING HORMONE FROM PREOPTIC-ANTERIOR HYPOTHALAMIC AND PITUITARY FRAGMENTS OF FEMALE GOLDFISH. [1]

6.1 INTRODUCTION

In goldfish brain and pituitary, two molecular forms of gonadotropin releasing hormone (GnRH) have been characterized by high performance liquid chromatography (HPLC) and radioimmunoassay (RIA) as salmon GnRH ([Trp^7, Leu^8]-GnRH, sGnRH) and chicken GnRH-II ([His^5, Trp^7, Tyr^8]-GnRH, cGnRH-II; see Chapter 8); the basic structure of GnRH (mammalian) is defined as pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. Using antisera raised against sGnRH, immunoreactive GnRH (ir-GnRH) neurons and nerve fibers have been found to be widely distributed in the goldfish brain, with the majority of cell bodies located in various forebrain areas, particularly the preoptic region (Kah *et al.*, 1986a). The direct innervation of pituitary gonadotrophs by GnRH peptidergic fibers originating in the preoptic region is thought to represent the neuroendocrine pathway for regulation of gonadotropin (GtH) secretion in goldfish and in teleosts in general (see Ball, 1981; Peter *et al.*, 1989b). In addition, the wide distribution of GnRH structures in different parts of the brain suggests that GnRH may also function as a neuromodulator or neurotransmitter, in addition to its hypophysiotropic function.

This Chapter describes the development of an *in vitro* system to study the release of GnRH from incubated slices of the preoptic-anterior hypothalamic (P-AH) region and

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fragments of the goldfish pituitary. In teleosts, the equivalent of the median eminence is incorporated into the rostral neurohypophysis of the pituitary (Batten and Ingleton, 1987) and immunoreactive GnRH (ir-GnRH) fibers have been shown to directly innervate the proximal pars distalis of the goldfish pituitary (Kah *et al*, 1986a). Thus, the pituitary fragments include the nerve terminals of the preoptic-hypophysial GnRH peptidergic neurons. The P-AH slices include the GnRH cell bodies in the ventral telencephalon and those of the preoptic-hypophysial GnRH pathway, and contain numerous GnRH nerve terminals, especially those in the ventral wall of the preoptic recess. The latter structure in goldfish is homologous to the organum vasculosum lamina terminalis (OVLT) in mammals (Wenger and Törk, 1968). HPLC and RIA were employed to characterize the GnRH released from the tissue preparations *in vitro*. In rats, *in vitro* studies using mediobasal hypothalamus (MBH) or median eminence (ME) tissue preparations have demonstrated calcium dependency of release induced by high K^+ concentrations (Rotsztejn *et al.*, 1976; Bigdeli and Snyder, 1978; Drouva *et al.*, 1981), and the release of GnRH in response to manipulation of ion channels (Drouva *et al.*, 1981) and second messenger systems (Ojeda *et al.*, 1979; Hartter and Ramirez, 1985; Ojeda *et al.*, 1985; Kim and Ramirez, 1986). In the present study, the GnRH release in response to several putative secretagogues, namely high K^+, calcium ionophore A23187, forskolin and prostaglandin E_2 (PGE_2) was examined for both P-AH slices and pituitary fragments of the goldfish.

Involvement of dopamine (DA; Chang *et al*, 1984, 1989; Chang *et al.*, submitted), norepinephrine (NE; Chang *et al.*, 1984) and serotonin (5-HT; Somoza and Peter, submitted; Somoza *et al.*, 1989) in the regulation of GtH secretion by actions at the level of the pituitary have been demonstrated in goldfish. Recent studies show that both brain and pituitary GnRH levels are altered by treatment with catecholamine synthesis inhibitors (Peter *et al.*, 1989b), raising the possibility that catecholamines may act at brain and pituitary sites to regulate GtH secretion through modulation of GnRH release.

However, the direct actions of monoamines on release of GnRH have not been studied in goldfish. In this study, the effects of DA, NE and 5-HT on *in vitro* GnRH release from the P-AH and pituitary of sexually mature female goldfish were also investigated.

6.2 MATERIALS AND METHODS

Animals and tissues

Goldfish, 7 - 10 cm standard length, were purchased from Grassyfork Fisheries Co. (Martinsville, IN) and Ozark Fisheries Inc. (Stoutland, MO). The fish were held in a 1800-liter flow-through aquarium at 15 ± 1°C on a simulated natural (Edmonton) photo-period for at least one week prior to use. Sexually mature female goldfish of 25-35 g body weight were used in the present study. Fish were anesthetized by immersion in 0.05% tricaine methanesulfonate (Syndel, Vancouver, BC) before sacrifice. The brain and pituitary were removed and the P-AH region (see Fig. 6.1) rapidly dissected from the brain on a glass petri dish. 250 µm slices were prepared from freshly collected P-AH using a McIlwain tissue chopper. Pituitary fragments were obtained by cutting the pituitary four times in different directions (each differing from the other by 90°) with the tissue chopper. The weight of tissue fragments of P-AH and pituitary was approximately 15 mg and 2 mg, respectively.

In vitro static incubation system

Freshly prepared slices or P-AH from 2 fish or fragments from four pituitaries were washed three times with one ml of incubation medium (pH 7.2) and preincubated in each well of the 24-well plate for 45 minutes in a controlled environmental chamber at 17°C. The incubation medium consisted of HEPES (25 mM)-buffered Hanks salt solution with glucose (1 mg/ml) and 0.1% bovine serum albumin (BSA); 60 mM ascorbic acid and 50

μM bacitracin were added to prevent degradation of monoamines and GnRH (McKelvy *et al.*, 1976) respectively. After the preincubation, the medium was replaced with one ml incubation medium containing the test substance or vehicle (control), and the tissue was incubated for 30 min. Medium containing verapamil, EGTA or no Ca^{2+} (Ca^{2+} omitted from the medium) was added to the wells during the preincubation period. Control tissue preparations incubated in medium alone were included in each 24-well plate. At the end of the incubation period, the medium was transferred to polypropylene culture tubes and frozen on dry ice until assayed for GnRH. The tissue recovered from the incubation well was sonicated in 1 ml of 2N acetic acid, and the supernatant obtained after centrifugation was lyophilized and stored until assay for GnRH. A 20 μl aliquot was taken from the homogenate prior to centrifugation for protein determination using Bradford's method (BioRad). Recovery of GnRH was evaluated by adding 100 pg of synthetic sGnRH (Peninsula Laboratories, Inc., Belmont, CA) to wells containing 1 ml incubation medium, with or without tissue.

Drugs and Chemicals

All buffer components and drugs were obtained from Sigma Chemical Co (St. Louis, MO). The following monoamines were used: dopamine-HCl, (-) arterenol (norepinephrine) HCl and serotonin creatinin sulfate. All monoamines were initially dissolved in acidified physiological saline and diluted to the desired concentration with incubation medium immediately prior to use. Stock solutions of A23187, forskolin, PGE_2 and (±) verapamil-HCl were prepared with ethanol and diluted with medium prior to use.

Radioimmunoassay of GnRH

GnRH released into the medium was measured by a RIA similar to that described previously (Chapter 3). A slight modification of the previous method was the use of Tris buffer at pH 7.2 in order to avoid adjustment of pH in the medium samples. Within assay

coefficient of variation was 10.2 % for samples at 2 pg/tube and 8.5% (n=6) for samples at 30 pg/tube. Interassay variation ranged from 13.8% (n=4) for 2 pg/tube and 12.2% (n=4) at 30 pg/tube. The minimum detectable level of the assay, defined as the mean plus two standard deviations for the reading of zero blank tubes was 0.20 ± 0.10 pg/tube (n=10). The doses at which relative percentage binding (B/Bo) equals 95% and 50% are about 0.5 and 15 pg, respectively. Synthetic sGnRH standards were made up in tubes containing incubation medium. This RIA, using antiserum PBL-49 at a final dilution of 1: 750,000, showed approximately 33% crossreaction to synthetic chicken GnRH-II (Peninsula Lab. Inc., Belmont, CA) compared to synthetic sGnRH standards (in Chapter 3).

Characterization of GnRH released in vitro

For RIA and HPLC analysis, samples of GnRH released spontaneously from P-AH and pituitary fragments *in vitro* were partially purified by loading onto C_{18} Sep-Pak cartriges (Waters Associates) and eluted with 50% acetonitrile in 0.25 M formic acid adjusted with triethylamine to pH 6.5 (TEAF). The volume of the final aqueous phase was reduced in SpeedVac (SAVANT) to about 1 ml. The soluble phase was filtered through a 0.22 μm Millipore GV filter and the volume adjusted to 1ml with Zenopure pure water (Quatra). The molecular forms of GnRH were separated by using a C_{18} reverse phase column (Supelco) as described in Chapter 8. Fresh standards consisting of a mixture of synthetic mammalian GnRH (mGnRH), lamprey GnRH ([Tyr^3, Leu^5, Glu^6, Trp^7, Lys^8]-GnRH), chicken GnRH-I ([Gln^8]-GnRH, cGnRH-I), cGnRH-II and sGnRH (Peninsula Lab. Inc., Belmont, CA) were chromatographed under similar conditions for comparison.

Statistical analysis

Statistical analyses were performed with Student's t-test and one way ANOVA followed by Duncan's multiple range test.

6.3 RESULTS

Release of GnRH from incubated P-AH slices and pituitary fragments was initially high during the 45 min preincubation period, then gradually decreased during the following six consecutive 30 min incubation periods (Fig. 6.2). High K^+ concentrations stimulated GnRH release from both P-AH slices and pituitary fragments during the first four consecutive 30 min incubation periods (Fig. 6.2).

Partially purified extracts of incubation medium were assayed in serial doubling dilutions for GnRH immunoactivity. Displacement curves of the GnRH released from P-AH slices and pituitary fragments paralleled that of the synthetic sGnRH (Fig. 6.3). As shown in Fig. 6.4, HPLC analysis of the medium extracts demonstrated that the major molecular form of the GnRH detected by the RIA was sGnRH . A minor peak in the HPLC fractions which coeluted with synthetic cGnRH-II is also recognized by the RIA (Fig. 6.4).

To assess the degradation of GnRH during incubation, 100 pg of synthetic sGnRH was added to incubation wells containing medium, with or without tissue. Recovery of exogenous sGnRH was 65% with P-AH slices and 80% with pituitary fragments without addition of bacitracin into the incubation medium; recovery was 81% (P-AH) and 92% (pituitary) in the presence of 50 μM bacitracin (data not shown).

No significant differences in tissue content before or after incubation were detected in either P-AH slices or pituitary fragments (Table 6.1). The amount of GnRH released from both tissue preparation during the 30 min incubation period represented less than 4% of the total initial content of both tissues. Total tissue GnRH and protein content of P-AH slices or pituitary fragments did not vary significantly among groups nor between experiments (data not shown). On this basis, the amount of GnRH released in subsequent experiments is expressed as pg GnRH released per 30 min incubation period.

As shown in Fig. 6.5, release of GnRH from both P-AH and pituitary fragments was stimulated by high K^+ (60 mM), forskolin (100 μM) and PGE_2 (1 μM). GnRH release from both tissue preparations was stimulated by high concentrations of K^+ (50 and 75 mM for P-AH, and 75 mM for pituitary; Fig. 6.6). Inhibition of calcium influx by omission of Ca^{2+} from the incubation medium (no Ca^{2+}) or by chelating the medium Ca^{2+} with 10^{-4} M EGTA did not affect GnRH release, but abolished high potassium stimulated release (Fig. 6.7). Verapamil, a voltage sensitive calcium channel blocker (1 μM), abolished the stimulation of GnRH release by high K^+ and calcium ionophore A23187 (1 μM; Fig. 6.8).

Fig. 6.9 shows the effects of DA, NE and 5-HT on the *in vitro* release of GnRH from the P-AH slices and pituitary fragments. GnRH release *in vitro* from incubated P-AH slices was inhibited by addition of DA (10 and 100 μM), but was stimulated by the addition of NE and 5-HT (10 and 100 μM). Release of GnRH from pituitary fragments was inhibited by DA (10 and 100 μM) and stimulated by 5-HT (1, 10 and 100 μM), but was not affected by NE.

6.4 DISCUSSION

In this study, the use of an *in vitro* static incubation system and RIA to study the release of GnRH from goldfish P-AH slices and pituitary fragments was validated. The combined HPLC and RIA study demonstrated that the major immunoreactive form of GnRH released from both P-AH slices and pituitary fragments *in vitro* is sGnRH. The presence of a second molecular form of GnRH immunoactivity in the HPLC fractions coeluting with synthetic cGnRH-II was also found. The observation of the *in vitro* release of both sGnRH- and cGnRH-II like peptides from the P-AH slices and pituitary fragments in the present study is consistent with the presence of both GnRH forms in these tissues (Chapter 8) and serum of goldfish (Peter *et al.*, 1989a). However, due to the

crossreactivity of the sGnRH antiserum with cGnRH-II (33%), the direct RIA measurements of the incubation medium in this study represent the total immunoreactivities by both sGnRH and cGnRH-II.

Release of GnRH from P-AH slices and pituitary fragments was stimulated by depolarizing concentrations of extracellular K^+. Release of GnRH from both tissues was also stimulated by forskolin (an adenylate cyclase stimulator) and PGE_2; these secretagogues have also been shown to stimulate *in vitro* release of GnRH from rat MBH (Ojeda *et al.*, 1979; Hartter and Ramirez, 1985; Ojeda *et al.*, 1985; Kim and Ramirez, 1986). Spontaneous and high K^+-stimulated GnRH release was observed during the first 2 hours of incubation, indicating the functional integrity of the secretory apparatus in the tissues over this time period. Attempts to use the whole P-AH and the whole pituitary *in vitro*, rather than fragments resulted in inconsistent GnRH release in response to high K^+ concentrations and PGE_2 (data not shown). This is presumably related to the low degree of oxygenation, and poor penetration of test substances into the whole tissue blocks.

It is generally believed that the release of neuropeptides is a calcium dependent process (Douglas, 1973). In this study, omission of Ca^{2+} from the incubation medium or chelating the remaining Ca^{2+} in medium with EGTA abolished the high K^+-stimulated GnRH release, but did not significantly reduce spontaneous release. The calcium ionophore A23187, which increases intracellular Ca^{2+} levels also stimulated GnRH release. The involvement of voltage sensitive calcium channels (VSCC) in GnRH release is demonstrated by the blockade of GnRH release induced by high K^+ or A23187 with verapamil, a VSCC blocker (Triggle and Janis, 1987). The dependency of hypothalamic GnRH release on Ca^{2+} *in vitro* has been demonstrated in rats (Rotsztejn *et al.*, 1976; Bigdeli and Snyder, 1978; Hartter and Ramirez, 1980). The inability of Ca^{2+} deficient medium or verapamil to significantly depress spontaneous GnRH release from goldfish P-AH slices and pituitary fragments *in vitro* in this study agrees with the results from

studies in rats (Drouva *et al.*, 1981), suggesting the independence of spontaneous release of GnRH from extracellular calcium.

This study is the first to demonstrate that GnRH is released *in vitro* from both P-AH slices and pituitary fragments in a teleost. The observation that P-AH slices, which contain both GnRH cell bodies and nerve terminals, actively release GnRH is not surprising. As discussed above (see Section 2.1, Introduction), immunocytochemical studies on the preoptic region of goldfish and teleosts revealed the presence of numerous ir-GnRH fibers (Kah *et al.*, 1986). The ventral wall of the preoptic recess, a homologue of the mammalian OVLT, is located outside the blood brain barrier and may represent an important site for GnRH release into the circulation of fish as has been suggested for mammals (Weindl and Sofroniew, 1978). A role of the GnRH structures in the OVLT in the control of the cyclic release of GtH in rats has been suggested (Samson and McCann, 1979; Wenger and Leonardelli, 1980).

The present results demonstrate that DA inhibits GnRH release *in vitro* from both P-AH slices and pituitary fragments of sexually mature female goldfish. Based on the ability of dopaminergic agents to alter the GnRH levels in discrete forebrain areas and the pituitary in normal male goldfish and in males undergoing spawning behavior with prostaglandin-treated females, we hypothesized previously in Chapter 4 that central DA has an inhibitory effect on the brain GnRH neuronal system. These results support and extend this hypothesis to show that DA can modulate GnRH neuronal activity by acting on the release processes at both the brain and pituitary levels in goldfish. A preoptic-hypophyseal dopaminergic pathway has been described (Kah *et al.*, 1984, 1986b, 1987) and suggested to mediate the dopaminergic inhibition of GtH secretion at the level of pituitary (see Peter *et al*, 1986). The close anatomical relationship between this dopaminergic pathway and the preoptic hypophyseal GnRH peptidergic pathway (Kah *et al.*, 1986a) provide potential for interaction of these systems at both the preoptic region and pituitary levels.

Nevertheless, the existence of synaptic contact between DA- and GnRH- containing neurons as reported in ewes (Kuljis and Advis, 1989) remains to be demonstrated in teleosts.

Evidence exists in mammals to suggest both a stimulatory and an inhibitory role for DA in the regulation of GnRH secretion (see Weiner *et al.*, 1988). The controversial effects of DA on *in vitro* GnRH release in rats have been explained by involvement of different DA pathways (MacKenzie *et al.*, 1984; James *et al.*, 1987) and receptors (Sarkar and Fink, 1981), and by the stimulation of endogenous NE release by DA (Jarjour *et al.*, 1986; Andersson *et al.*, 1988). In goldfish, concomitant stimulation of NE release by DA would not affect the inhibitory effects of DA on GnRH release from the pituitary as NE produced no significant effects on GnRH release at this level; however, release of NE by DA will attenuate the inhibitory effects of DA on GnRH release from the P-AH since NE stimulates the GnRH release. Investigation of specific DA receptor subtypes in the regulation of GnRH secretion is described in Chapter 7.

In the present study, NE (10-100 μM) stimulated GnRH release from the P-AH slices but not from pituitary fragments *in vitro* in goldfish, suggesting different adrenergic control mechanisms for GnRH release at these two loci. In contrast, NE has stimulatory actions on GnRH release from rat ME, but not MBH in an *in vitro* static incubation system (Rotszteyn *et al.*, 1977; Negro-Vilar *et al.*, 1979). However, stimulatory effects of NE on GnRH release from the MBH have been found using an *in vitro* superfusion system in rats (Nowak and Swerdloff, 1985; Jarjour *et al.*, 1986) and Japanese quail (Millam *et al.*, 1984).

This study also demonstrated that 5-HT stimulates *in vitro* release of GnRH from both P-AH slices and pituitary fragments. The results agree with the observed stimulatory actions of 5-HT on GtH release from goldfish pituitary *in vivo* (Somoza *et al.*, 1988) and *in vitro* (Somoza and Peter, submitted). However, a stimulatory role of 5-HT on GnRH

secretion is not supported by the results of previous studies showing that injection of 5-HT into third ventricle of the female goldfish brain does not affect serum GtH levels half an hour after treatment (Somoza *et al.*, 1988). Further studies are required to resolve this apparent discrepancy. Nevertheless, direct innervation of the pars distalis of the pituitary by immunoreactive 5-HT fibers has been demonstrated in goldfish (Kah and Chambolle, 1983), as well as in other teleosts (Ekström and van Veen, 1984; Margolis-Kazan *et al*, 1985). In rats, synaptic contact of 5-HT containing nerve terminals with GnRH neurons in the preoptic region has been demonstrated (Kiss and Halasz, 1985). Using a double chamber perifusion of the ME and pituitary obtained from proestrus rats, it has been shown that 5-HT can stimulate GnRH release in a dose dependent manner (Vitale *et al.*, 1986). In another *in vitro* study, 5-HT also increased the frequency of release from hypothalami obtained from estrogen treated ovariectomized (OVX) rats, but had no effect on hypothalami from OVX rats (Meyer, 1989), suggesting a dependency of the 5-HT actions on sex steroids. In contrast, Charli *et al* (1978) showed that 5-HT inhibits release of GnRH from MBH, but not from the OVLT.

In conclusion, an *in vitro* incubation system has been developed for the study of GnRH release from P-AH slices and pituitary fragments in goldfish. The characteristics of the *in vitro* GnRH release in terms of the stimulation by various secretagogues (high K^+, calcium ionophore, forskolin and PGE_2) and the calcium dependency of the high K^+-induced release are analogous to those described in rats (Rotsztejn *et al.*, 1976; Bigdeli and Snyder, 1978; Ojeda *et al.*, 1979; Drouva *et al.*, 1981, Hartter and Ramirez, 1985; Ojeda *et al.*, 1985; Kim and Ramirez, 1986) and in birds (Japanese quail: Millam *et al.*, 1984), suggesting conserved control mechanisms for GnRH release in vertebrates. This study also demonstrates, for the first time in a teleost, that GnRH release from the P-AH and pituitary is modulated by various monoamines, suggesting that monoamines could act at different levels of the neural circuity for the regulation of pituitary GtH secretion in goldfish (see Peter *et al*, 1986). The development of an *in vitro* approach to investigate the direct

actions of various neural factors on GnRH release in this study will enhance our understanding of the neural regulation of GnRH neuronal activity as well as pituitary GtH secretion in goldfish and in other teleosts.

Table 6.1
Tissue GnRH content and percent release during *in vitro* static incubation.

Tissue	GnRH content (pg/mg protein)		GnRH released into medium (% of total tissue content)
	Pre-incubation	Post-incubation	
Preoptic-anterior hypothalamus	0.768 ± 0.070	0.837 ± 0.059	3.97 ± 0.52
Pituitary	2.64 ± 0.26	2.53 ± 0.23	3.18 ± 0.48

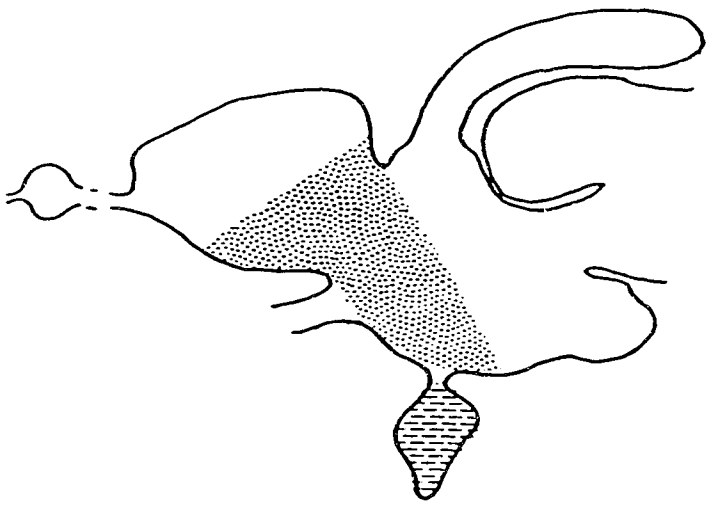

Fig. 6.1. Diagrammatic sagittal section of goldfish forebrain showing the preoptic-anterior hypothalamus-containing brain area (shaded) and pituitary (hatched) dissected for the *in vitro* static incubation.

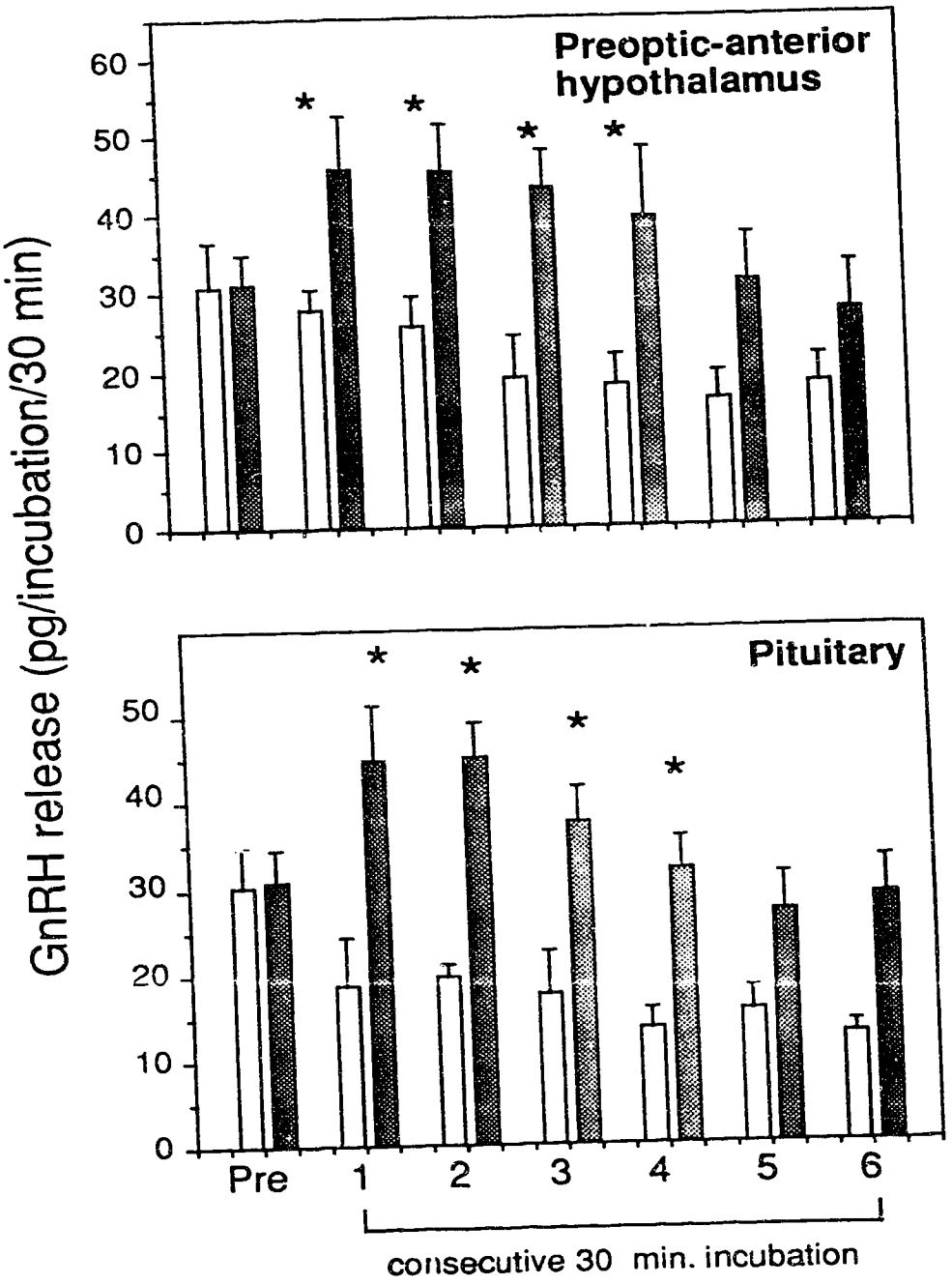

Fig. 6.2. Effect of 60 mM K^+ on release of GnRH from preoptic-anterior hypothalamic slices and pituitary fragments *in vitro* during 45 min preincubation (Pre) and 6 consecutive 30 min incubation periods. Medium was replaced at the end of the preincubation and each subsequent incubation period. Each bar represents the mean ± SEM (n=8). Shaded and empty columns represent 60 mM K^+ and control groups respectively. * indicates a significant difference ($p < 0.05$, Student's t-test) from the control group.

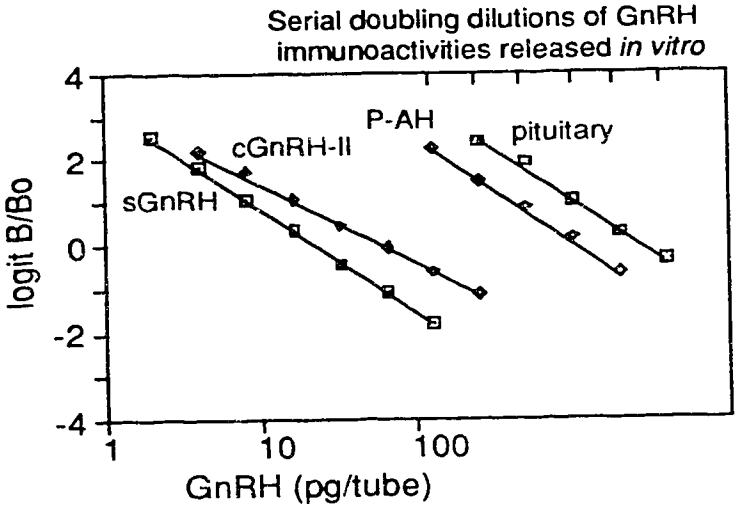

Fig. 6.3. Displacement of ^{125}I-sGnRH from antiserum PBL-49 by synthetic sGnRH, cGnRH-II and the partially purified GnRH immunoreactive materials released from preoptic-anterior hypothalamic (P-AH) slices and pituitary fragments *in vitro*. Each point represents the average of duplicate determinations.

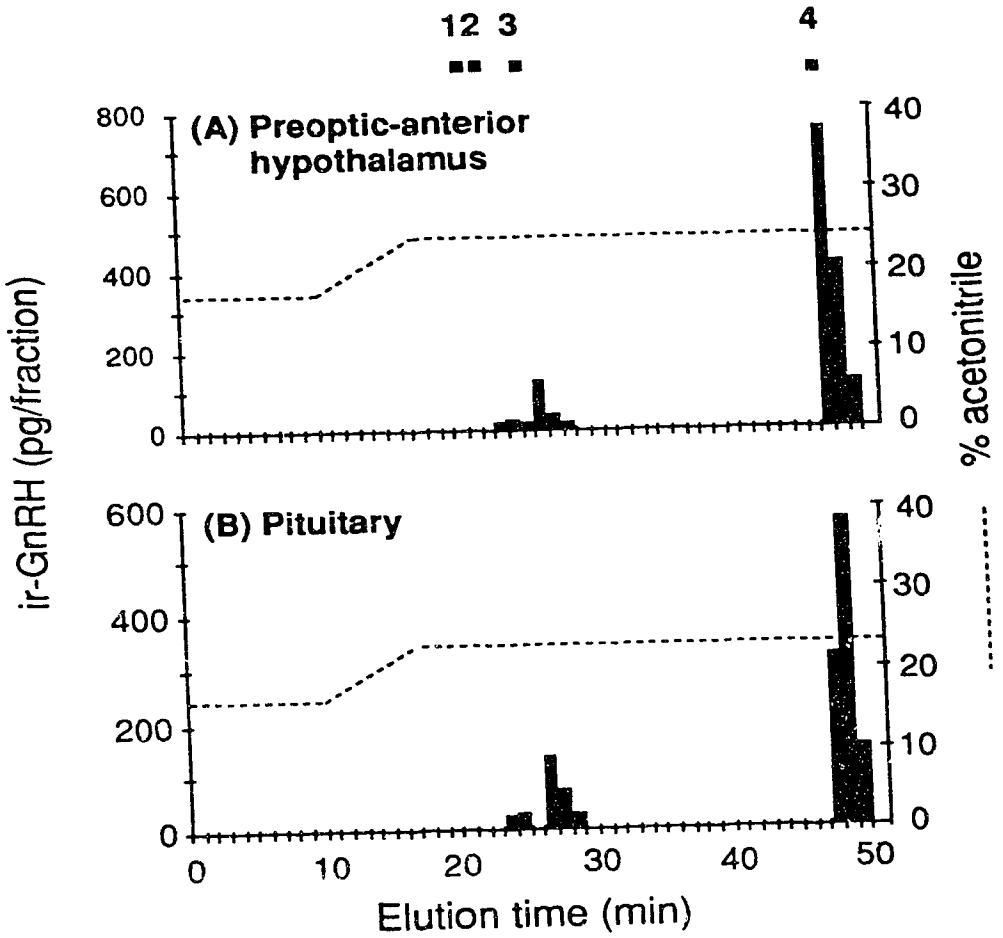

Fig. 6.4. Reverse phase HPLC of GnRH immunoactivities released from goldfish preoptic-anterior hypothalamic slices (A) and pituitary fragments (B) *in vitro*. Elution positions of mGnRH and lamprey GnRH (1), cGnRH-I (2), cGnRH-II (3), and sGnRH (4) are indicated. Each column represents the total amount (mean of duplicate determinations) of ir-GnRH detected in each 1 ml fraction (flow rate 1 ml/min). The mobile phase was acetonitrile in TEAF (pH 6.5). Percent acetonitrile is shown as a dotted line.

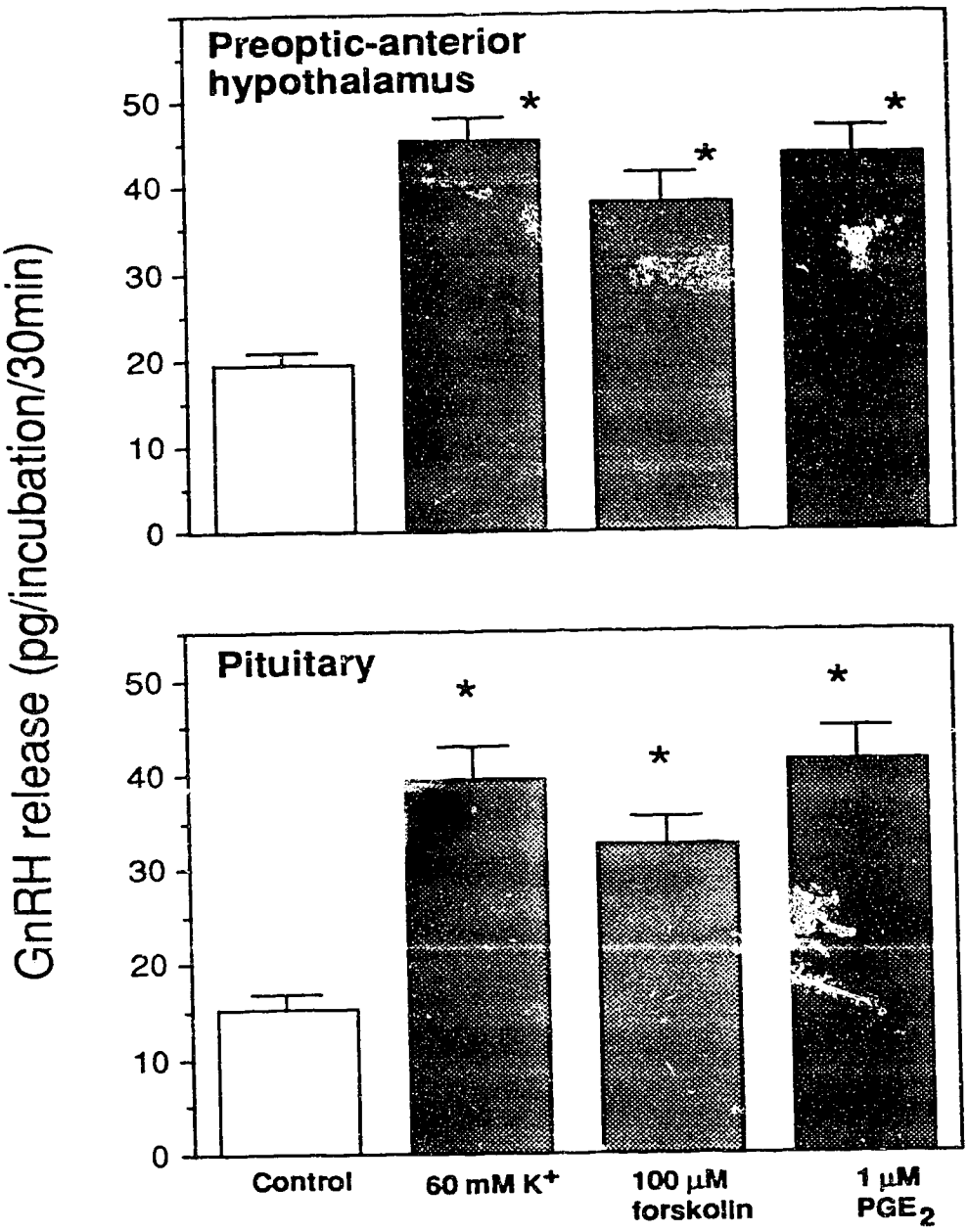

Fig. 6.5. Effects of 60 mM K^+, forskolin and PGE_2 on spontaneous GnRH release from preoptic-anterior hypothalamic slices and pituitary fragments of sexually mature female goldfish *in vitro*. Each column represents mean ± SEM (n=12). * indicates a significant difference ($p < 0.05$, one-way ANOVA and Duncan's multiple range test) from the control group.

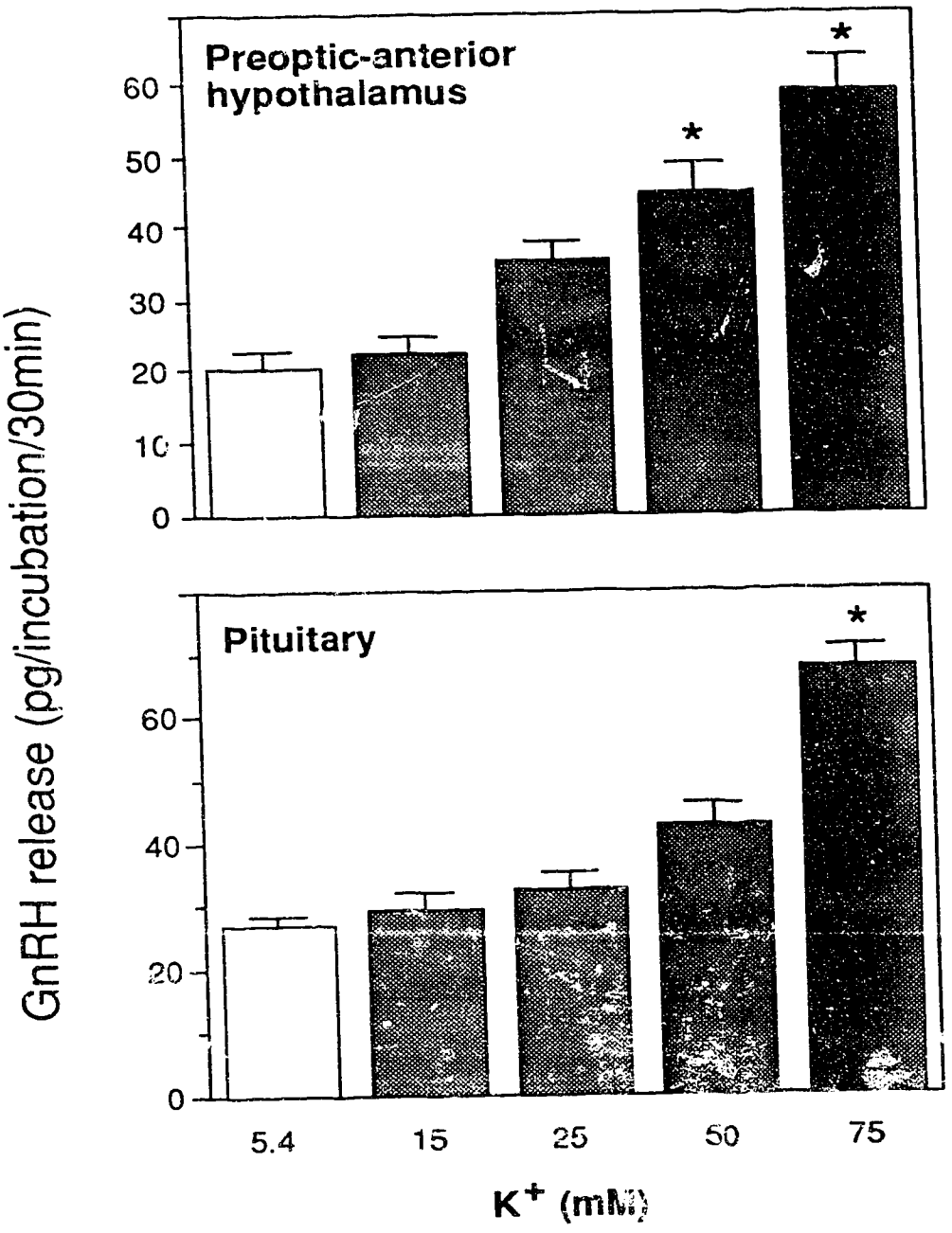

Fig. 6.6. Effects of different concentrations of K^+ on GnRH release from preoptic-anterior hypothalamic slices and pituitary fragments *in vitro*. Each column represents mean ± SEM (n=8). * indicates a significant difference ($p < 0.05$, one-way ANOVA and Duncan's multiple range test) from the control group.

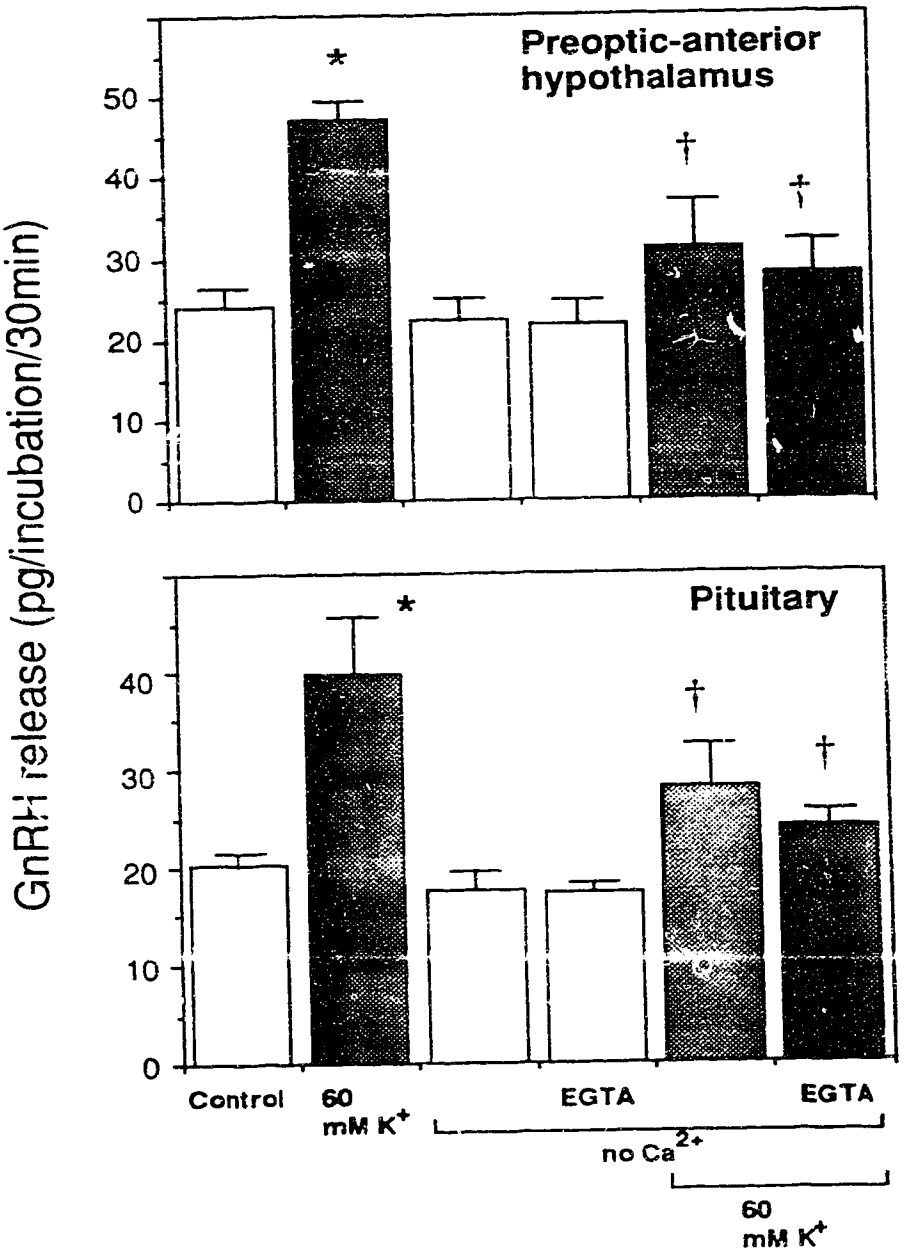

Fig. 6.7. Calcium dependency of K^+-induced GnRH release from preoptic-anterior hypothalamic slices and pituitary fragments *in vitro*. Tissues were incubated in medium in the presence or absence of Ca^{2+} (1.3 mM), EGTA (0.1 mM) or K^+ (60 mM). Each column represents mean ± SEM (n=8). * indicates a significant difference ($p < 0.05$, one-way ANOVA and Duncan's multiple range test) from the control. † indicates a significant difference ($p < 0.05$, Student's t-test) from the corresponding 60 mM K^+ groups.

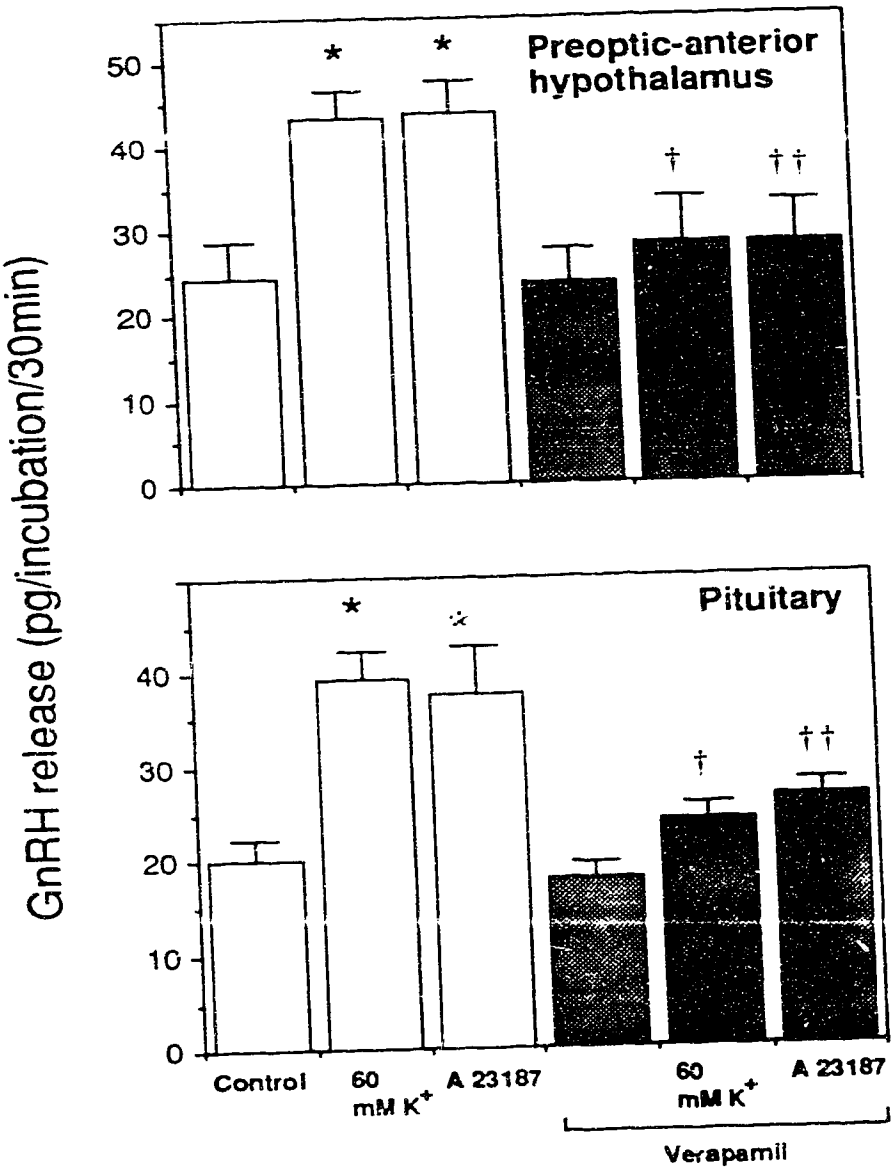

Fig. 6.8. Inhibition by verapamil (1 μM) of GnRH release stimulated by K^+ (60 mM) and A23187 (1 μM) from preoptic-anterior hypothalamic slices and pituitary fragments in vitro. Each column represents mean ± SEM (n=8). * indicates a significant difference ($p < 0.05$, one-way ANOVA and Duncan's multiple range test) from the control group. † and †† indicate significant differences ($p < 0.05$, Student's t-test) from the 60 mM K^+ groups and A23187 groups respectively.

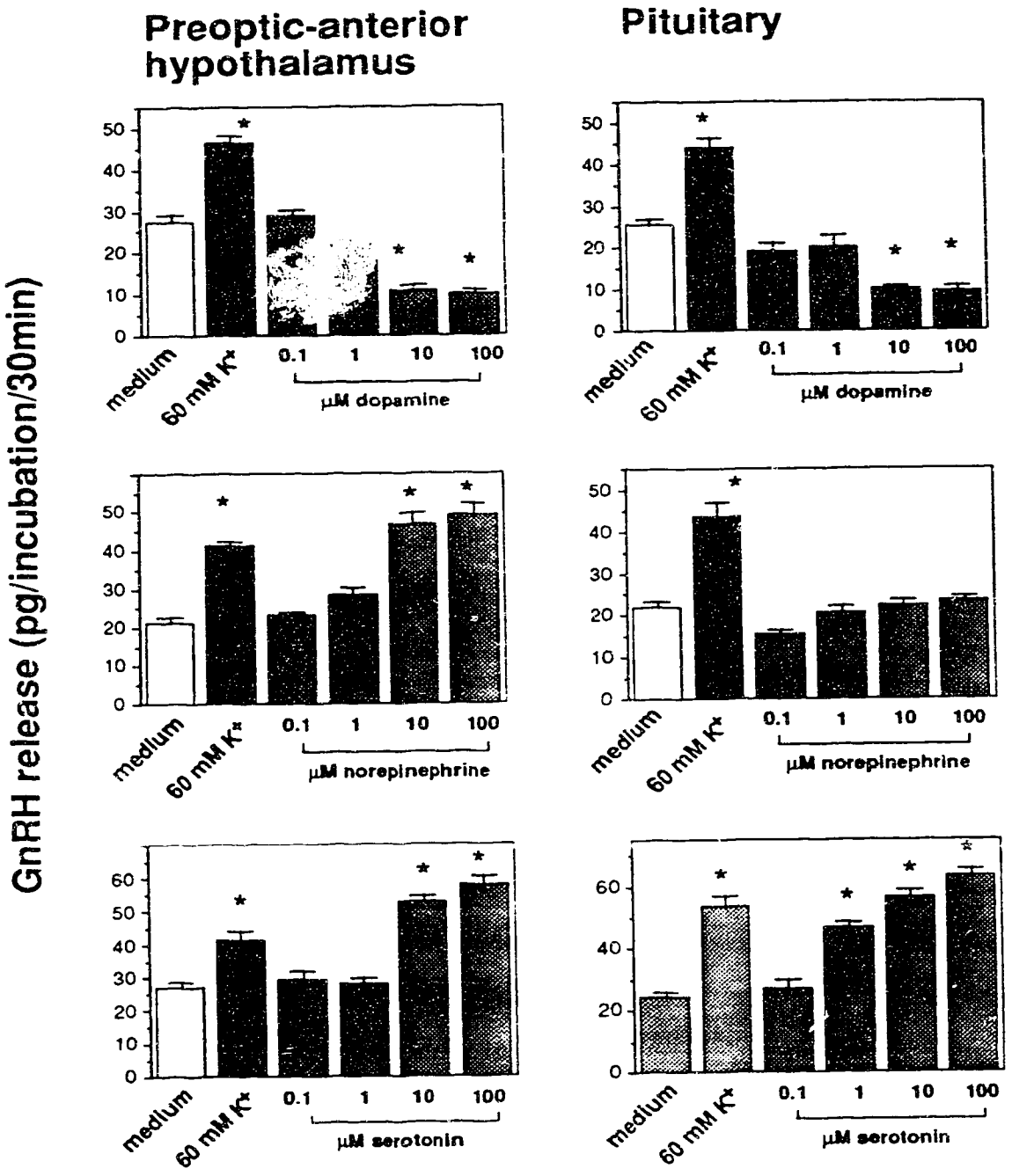

Fig. 6.9. Effects of different concentrations of DA, NE and 5-HT on GnRH release from preoptic-anterior hypothalamic slices and pituitary fragments of sexually mature female goldfish *in vitro*. Each column represents mean ± SEM (n=8). * indicates a significant difference ($p < 0.05$, one-way ANOVA and Duncan's multiple range test) from the respective control group.

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7. ACTIONS OF ADRENERGIC AND DOPAMINERGIC RECEPTOR SUBTYPES ON RELEASE OF GONADOTROPIN-RELEASING HORMONE FROM GOLDFISH PREOPTIC-ANTERIOR HYPOTHALAMUS AND PITUITARY *IN VITRO* [1]

7.1 INTRODUCTION

Involvement of catecholamines in the regulation of gonadotropin (GtH) secretion in teleosts is well documented (see Peter *et al.*, 1986). Results of studies in goldfish demonstrate that dopamine (DA) has direct inhibitory effects, whereas norepinephrine (NE) has direct stimulatory effects on GtH secretion at the level of the pituitary (Chang *et al.*, 1984; Chang *et al.*, submitted). Recent studies showed that gonadotropin-releasing hormone (GnRH) levels in the brain and pituitary of goldfish are altered by catecholamine synthesis inhibitors, suggesting direct actions of catecholamines on brain GnRH neuronal activity (Peter *et al.*, 1989). Results presented in Chapter 6 showed that NE stimulates GnRH release from incubated preoptic-anterior hypothalamus (P-AH) slices of sexually mature female goldfish *in vitro*. On the other hand, DA acts at both the preoptic-anterior hypothalamus and pituitary level to inhibit release of GnRH *in vitro* (in Chapter 6). Thus, it is apparent that NE and DA can act at multiple sites to modulate both GnRH and GtH secretion. The aim of this study was to investigate the effects of NE and DA on GnRH release from preoptic-anterior hypothalamus and pituitary fragments of sexually mature male goldfish *in vitro*. To study the specificity of the action of NE and DA on *in vitro*

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[1] A version of this chapter has been submitted for publication: Yu, K. L., and Peter, R. E. Brain Res.

GnRH release, the adrenergic and dopaminergic receptor subtypes involved were characterized using specific agonists and antagonists.

7.2 MATERIALS AND METHODS

Animals and tissues

Goldfish, 7 - 10 cm standard length, were purchased from Grassyfork Fisheries Co. (Martinsville, IN) and Ozark Fisheries Inc. (Stoutland, MO). The fish were held in an 1800-liter flow-through aquarium at 15 ± 1°C on a simulated natural (Edmonton) photoperiod for at least one week prior to use. Sexually mature male goldfish of 25-35 g body weight were used in the present study. Fish were anesthetized by immersion in 0.05% tricaine methanesulfonate (Syndel, Vancouver, BC) before handling. The brain and pituitary were removed and areas containing the preoptic-anterior hypothalamus were rapidly dissected from the brain on a glass petri dish, as described previously in Chapter 6. 250 μm slices of the preoptic-anterior hypothalamus were prepared using a McIlwain tissue chopper. Pituitary fragments were obtained by cutting the pituitary four times in different directions (each differs from other by 90°) with a tissue chopper.

In vitro static incubation system

Incubation of preoptic-anterior hypothalamus (two per well) and pituitary (four per well) fragments was performed in 24-well plates, with 1 ml of Hepes-buffered Hanks salt medium (pH 7.2) at 17 °C as described in Chapter 6. The medium contains glucose (1 mg/ml), 0.1% bovine serum albumin (BSA), 60 mM ascorbic acid and 50 μM bacitracin. Tissue fragments were washed three times with 1 ml of medium. In all cases, the tissue fragments were preincubated for 45 min, followed by a 30-min incubation period. Control and high K^+ (60 mM) group are included in each experiment for comparison with the test

group. When receptor antagonists were used, they were added to both preincubation and incubation media. At the end of the incubation period, the medium was transferred to polypropylene culture tubes and frozen on dry ice until assay for GnRH.

Preparation of drugs

(-) Arterenol HCl , DA-HCl, phenylephrine HCl, isoproterenol, yohimbine HCl (Sigma, St. Louis, MO.), clonidine HCl (Catapres, a gift from Boehringer-Ingelheim Ltd., Ridgefield, CT), phentolamine HCl (Rogitine, a gift from Ciba-Geigy Canada Ltd., Dorval, Quebec) and prazosin HCl (a gift from Pfizer Canada Inc., Dorval, Quebec) were dissolved in acidified physiological saline and diluted with incubation medium immediately prior to use. Stock solutions of apomorphine HCl (Sigma, St. Louis, MO.), SKF-38393, SKF-83566 (Research Biochemicals Inc., Wayland, MA.), LY-171555 (quinpirole HCl, a gift from Eli Lilly & Co., Indianapolis, IN), pimozide and domperidone (gifts from Janssen Pharmaceutica, Beerse, Belgium) were prepared in dimethyl sulfoxide and diluted with incubation medium immediately prior to use.

Radioimmunoassay of GnRH

GnRH released into the medium was measured by RIA according to a procedure described in Chapter 6. Two molecular forms of GnRH, corresponding to salmon GnRH ([Trp^7, Leu^8]-GnRH, sGnRH) and chicken GnRH-II ([His^5, Trp^7, Tyr^8]-GnRH, cGnRH-II), have been shown to be released from both the preoptic-anterior hypothalamus and pituitary of goldfish *in vitro* (Chapter 6). The antiserum PBL-49 used in the RIA shows 33% crossreaction to synthetic cGnRH-II compared to synthetic sGnRH (see Chapter 3). HPLC analysis showed that sGnRH constitutes most of the crossreactive material in the incubated medium measured by this RIA (Chapter 6). Synthetic sGnRH (Peninsula Laboratories, San Carlos, CA) is used for the standards in the RIA; the results

are expressed as picograms of GnRH released per incubation per 30 min.

Statistical analysis

The statistical significance of the differences observed was determined by using Student's t-test and one way ANOVA followed by Duncan's multiple range test.

7.3 RESULTS

Effects of NE and adrenergic receptor agonists on GnRH release from preoptic-anterior hypothalamic slices and pituitary fragments.

Figure 7.1. shows the effects of NE (0.01 to 100 μM) on GnRH release. At 10 μM and 100 μM NE, a significant increase in the release of GnRH from P-AH slices was obtained. On the other hand, none of the concentrations of NE tested elicited a significant change in the amount of GnRH released from pituitary fragments during the 30 min incubation period.

The effects of some adrenergic receptor agonists (α_1, α_2, β) on the release of GnRH were tested at a concentration of 1 μM (Fig. 7.2). NE (60 μM) and phenylephrine (α_1 agonist) significantly stimulated GnRH release from P-AH fragments, but not from pituitary fragments. Clonidine (α_2 agonist), isoproterenol (β agonist), and lower doses of phenylephrine (0.001 to 0.1 μM, data not shown) had no significant effects on GnRH release from P-AH slices and pituitary fragments.

Effects of adrenergic antagonists on spontaneous and NE-induced GnRH release from preoptic-anterior hypothalamic slices.

Four adrenergic receptor antagonists were used at 1 μM: phentolamine (α_1/α_2 antagonist), prazosin (α_1 antagonist), yohimbine (α_2 antagonist) and propranolol (β antagonist). None of the adrenergic antagonists tested had a significant effect on

spontaneous GnRH release (Fig. 7.3). On the other hand, phentolamine and prazosin abolished the stimulatory effects of NE on GnRH release from P-AH slices; yohimbine and propranolol were ineffective (Fig. 7.3).

Effects of DA and dopaminergic receptor agonists on GnRH release from preoptic-anterior hypothalamic slices and pituitary fragments.

Figure 7.4 shows the effects of DA (0.01 to 100 μM) on GnRH release from P-AH slices and pituitary fragments. DA significantly reduced GnRH released from both tissue preparations at concentrations of 10 and 100 μM.

Dopaminergic receptor agonists (D_1, D_2) were tested at 1 μM on the release of GnRH from both P-AH slices and pituitary fragments. As shown in the upper panel of Fig. 7.5, 10 μM DA, apomorphine (D_1/D_2 agonist) and SKF-38393 (D_1 agonist) significantly reduced GnRH release from P-AH slices; bromocriptine and LY-171555 (D2 agonists) were ineffective. On the other hand, apomorphine and SKF-38393 had no significant effects on GnRH release from pituitary fragments, while the D_2 agonists bromocriptine and LY-171555 inhibited GnRH release (lower panel of Fig. 7.5).

Effects of dopaminergic receptor antagonists on spontaneous and DA-induced inhibition of GnRH release from preoptic-anterior hypothalamic slices and pituitary fragments.

Three DA-receptor antagonists were used at 1 μM: SKF-83566 (D_1 antagonist), pimozide (D_2>D_1 antagonist) and domperidone (D_2 antagonist). SKF-83566 significantly inhibited spontaneous GnRH release from P-AH slices; whereas other DA antagonists had no significant effects (upper panel of Fig. 7.6). None of the DA antagonists blocked the inhibitory effects of DA on GnRH release from P-AH slices (upper panel of Fig. 7.6).

None of the DA antagonists tested had a significant effect on release of GnRH from pituitary fragments (lower panel of Fig. 7.6). Pimozide and domperidone blocked and

reversed, respectively, the inhibitory effects of DA on GnRH release from pituitary fragments; the D_1 antagonist SKF-83566 was ineffective in blocking the effects of DA (lower panel of Fig. 7.6).

7.4 DISCUSSION

In agreement with previous studies using P-AH slices and pituitary fragments from sexually mature female goldfish (in Chapter 6), this study demonstrates that NE stimulates GnRH release from P-AH slices, whereas DA inhibits GnRH release from both P-AH slices and pituitary fragments of sexually mature male goldfish. These results suggest that similar adrenergic and dopaminergic mechanisms for the modulation of GnRH secretion in the P-AH and pituitary exist in both sexes of goldfish.

Four classes of adrenergic receptors have been described in the central nervous system of mammals: α_1- and α_2- (U'Prichard and Snyder, 1979) and β_1- and β_2-adrenergic receptors (Minneman *et al.*, 1979). In the present study, the stimulatory effects of NE on GnRH release from P-AH slices of goldfish were mimicked by the specific α_1 agonist phenylephrine. This stimulatory effect of NE was blocked by phentolamine (α_1/α_2 antagonist) and prazosin (α_1 antagonist), but not by α_2- or β- receptor antagonists. These results suggest that the NE-induced stimulation of GnRH release is mediated by α_1-adrenergic receptors in the P-AH of goldfish. α-adrenergic receptors have been shown to mediate the NE-stimulation of *in vivo* (rats: Sarkar and Fink, 1981; rhesus monkey: Terasawa *et al.*, 1988, Pau *et al.*, 1989) and *in vitro* (Negro-Vilar *et al.*, 1979; Ojeda *et al.*, 1982; Nowak and Swerdloff, 1985) GnRH release from mediobasal hypothalamus (MBH) or median eminence (ME) in rats. However, the involvement of specific α–receptor subtypes in NE-induced GnRH release is controversial. Heaulme and Dray (1984) showed that NE stimulates GnRH release from the rat ME through α_1-

receptors whereas α_2-receptors have been implicated by others (Negro-Vilar, 1982). In Japanese quail, the β-adrenergic agonist isoproterenol, similar to NE, stimulates GnRH release from hypothalamus *in vitro* (Millam *et al*, 1984).

The involvement of specific DA-receptor subtypes in the inhibition of GnRH release from the P-AH and pituitary of sexually mature male goldfish has also been demonstrated. It is generally accepted that there are two distinct types of DA-receptors in the brain: D_1 and D_2 (Creese *et al.*, 1983; Stoff and Kebabian, 1984). D_1-receptors are solely post-synaptic, while D_2-receptors are located both pre- and post-synaptically (Stoff and Kebabian, 1984). In the present study in goldfish, it was found that the selective D_1 agonist SKF-38393 (Setler *et al.*, 1978; O'Boyle and Waddington, 1984) and the nonselective D_1/D_2 agonist apomorphine mimicked the inhibitory actions of DA on GnRH release from P-AH slices. This result suggests that D_1-receptors mediate the inhibitory effects of DA on GnRH release from the P-AH. However, it is paradoxical that the D_1 antagonist SKF-83566 did not block the inhibitory effects of DA on GnRH release from P-AH slices and that SKF-83566 by itself inhibited spontaneous GnRH release from P-AH slices. This paradoxical effect of SKF-83566 may be related to its 5-HT_2-receptor blocking activity (Bischoff *et al.*, 1986, McQuade *et al.*, 1988); indeed, 5-HT has been shown to be stimulatory to GnRH release from P-AH in goldfish (Chapter 6).

In contrast to the involvement of D_1-receptors in the inhibition of GnRH release from the goldfish preoptic-anterior hypothalamus, D_2-receptors appear to mediate the DA-induced inhibition of GnRH release from nerve terminals in the pituitary. The selective D_2 agonists bromocriptine (Creese *et al.*, 1983) and LY-171555 (Tsuruta *et al.*, 1981), but not the D_1 agonist SKF-38393, inhibit GnRH release from pituitary fragments. The selective D_2 antagonist, domperidone (Lazareno and Nahorski, 1982) and pimozide (Creese *et al.*, 1983) reversed the inhibition of GnRH release by DA; the D_1 antagonist SKF-83566 was ineffective. These results suggest that the inhibition of GnRH release from the pituitary of goldfish by DA is mediated by D_2-, rather than D_1-receptors.

However, the observation that DA stimulated GnRH release from pituitary fragments in the presence of a selective D_2 antagonist reveals a complicated DA mechanism. One interpretation is that blockade of D_2-receptor sites by domperidone allows subsequent stimulation of GnRH release through activation of D_1-receptors. A stimulatory role of D_1-receptors on GnRH release from the pituitary may explain the inability of the nonselective D_1/D_2 agonist apomorphine to inhibit GnRH release in this study. However, the inability of the D_1 agonist SKF-38393 (1 μM) to stimulate GnRH release from the pituitary fragments argues against this idea. Since a single concentration of drugs was used in this study, further investigations are necessary to elucidate fully the contribution of each DA-receptor subtype in the modulation of GnRH release from the pituitary of goldfish. Interaction of D_1- and D_2- receptors in the expression of several behavioral and electrophysiological phenomena in the central nervous system has been demonstrated recently in rats (Braun and Chase, 1986; Walters *et al.*, 1987; White, 1987).

Unlike the situation in goldfish in which predominantly inhibitory effects of DA on GnRH release from both the P-AH and pituitary have been demonstrated, there is evidence to suggest both stimulatory and/or inhibitory actions of DA in the regulation of GnRH secretion in rats (see Weiner *et al.*, 1988). Inhibitory effects of DA on *in vivo* (Sarkar and Fink, 1981) and *in vitro* release of GnRH from the hypothalamus of female rats have been described (Tasaka *et al.*, 1985). Sarkar and Fink (1981) suggested that the inhibitory actions of DA on GnRH release are mediated by receptors blocked by pimozide and domperidone (D_2 antagonists), while the stimulatory actions of DA are mediated by receptors blocked by haloperidol (D_2>D_1 antagonist). On the other hand, Fuxe *et al* (1977, 1988) proposed that D_1-receptors are involved in the inhibitory actions of the tubero-infundibular DA pathway on GnRH secretion. The presence of D_1-receptors in the ME has been demonstrated in rats (Fuxe *et al.*, 1983). On the other hand, DA appears to stimulate GnRH release from MBH or ME *in vitro* in male rats (Rotsztejn *et al.*, 1977;

Negro-Vilar *et al.*, 1979). Studies in ovariectomized estrogen-treated rats suggest that D_1 receptors mediate the stimulatory effects of the incerto-hypothalamic DA tract on GtH secretion, presumably through modulation of GnRH release (MacKenzie *et al.*, 1984; James *et al.*, 1987). Alternatively, the stimulatory effects of DA on GnRH release from MBH in rats *in vitro* have been suggested to be mediated by release of NE induced by activation of D_1-DA receptors (Jarjour *et al.*, 1986; Andersson *et al.*, 1988a,b)

In conclusion, this study demonstrates that NE and DA act through specific receptors to modulate *in vitro* release of GnRH from the P-AH and pituitary in sexually mature male goldfish. The stimulatory effects of NE on GnRH release appear to be localized in the P-AH and mediated by specific α_1-adrenergic receptors. On the other hand, the inhibitory actions of DA on GnRH release from the P-AH region and pituitary involve two pharmacologically distinct DA receptors: D_1- and D_2- receptors, respectively. The relative physiological importance of the adrenergic and dopaminergic receptor subtypes in the regulation of GnRH secretion from the P-AH region and pituitary, and on pituitary GtH secretion in goldfish, remains to be studied in the future.

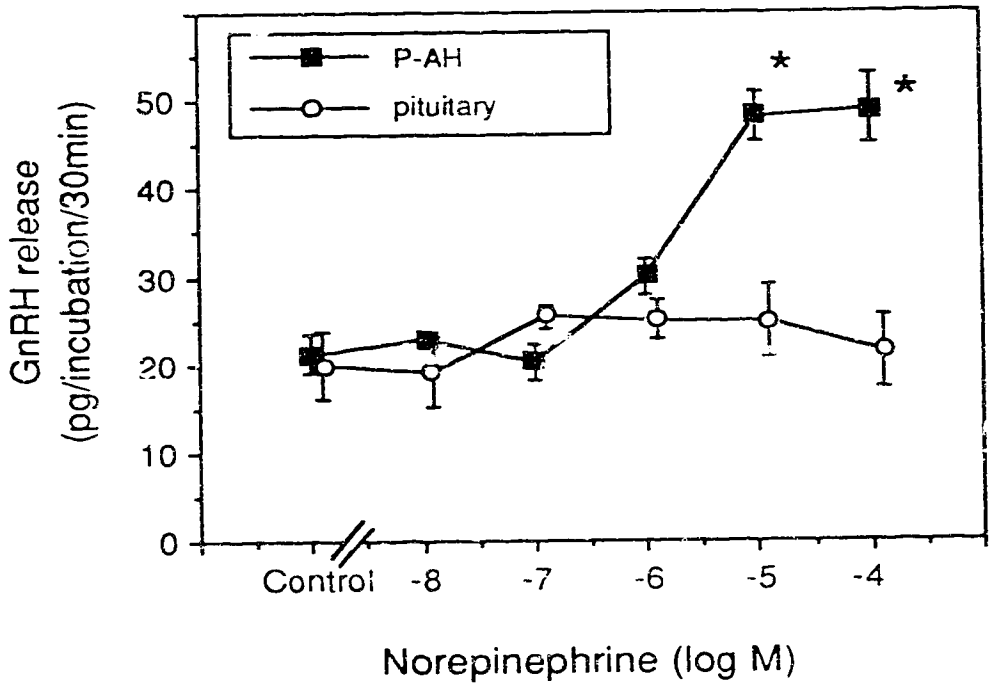

Fig. 7.1. Dose-response relationship between NE concentrations and GnRH release from preoptic-anterior hypothalamic (P-AH) slices and pituitary fragments of sexually mature male goldfish *in vitro*. Tissues were preincubated in medium for 45 min followed by a 30 min incubation period in the presence or absence of test substances. Values represents mean ± SEM (n=8). * indicates a significant difference ($p < 0.05$, one-way ANOVA and Duncan's multiple range test) from the control group.

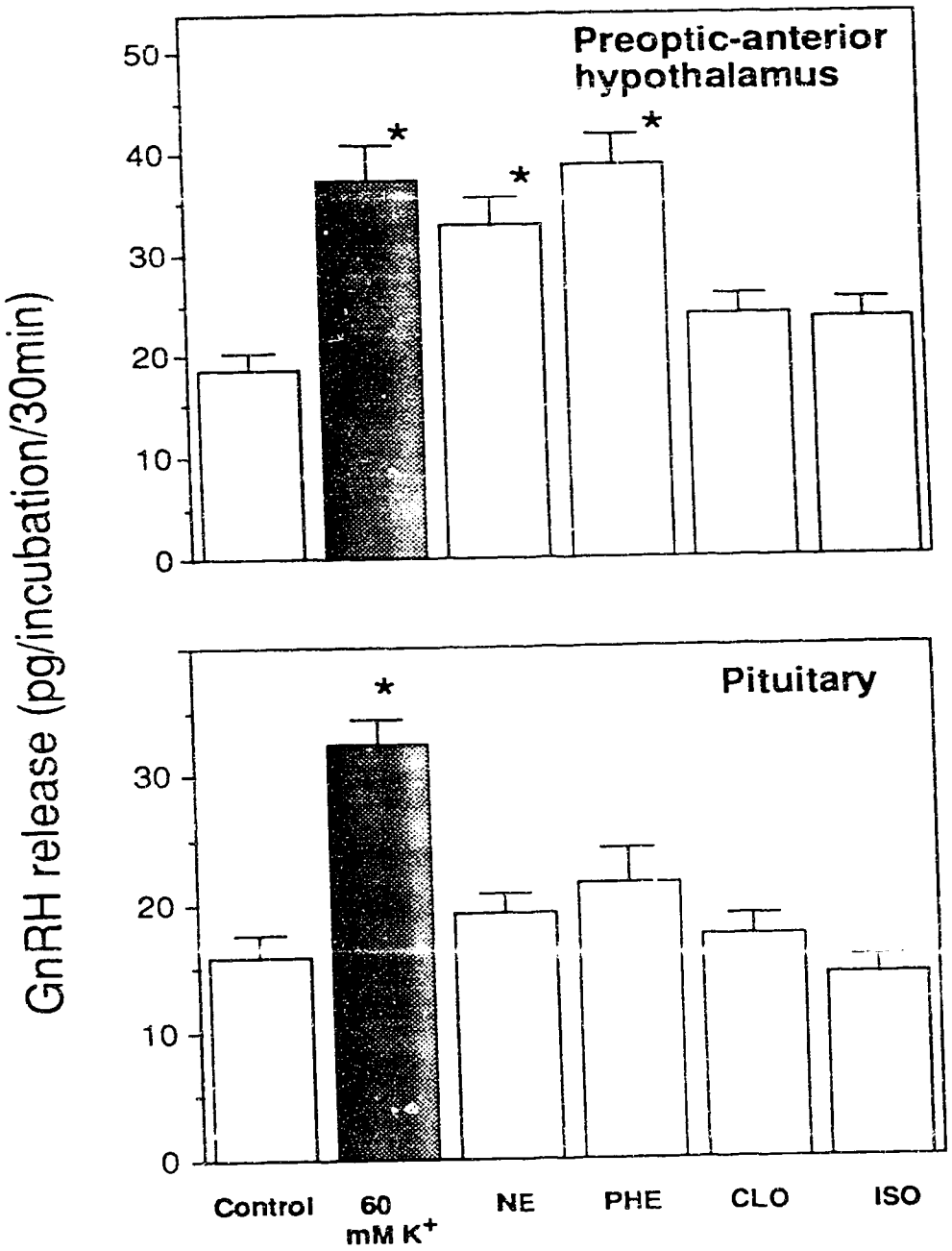

Fig. 7.2. Effects of NE (60 μM) and the adrenergic receptor agonists phenylephrine (PHE), clonidine (CLO) and isoproterenol (ISO, all at 1 μM) on the release of GnRH from preoptic-anterior hypothalamic slices and pituitary fragments of sexually mature male goldfish *in vitro*. Each column represents mean ± SEM (n=8). * indicates a significant difference ($p < 0.05$, one-way ANOVA and Duncan's multiple range test) from the control group.

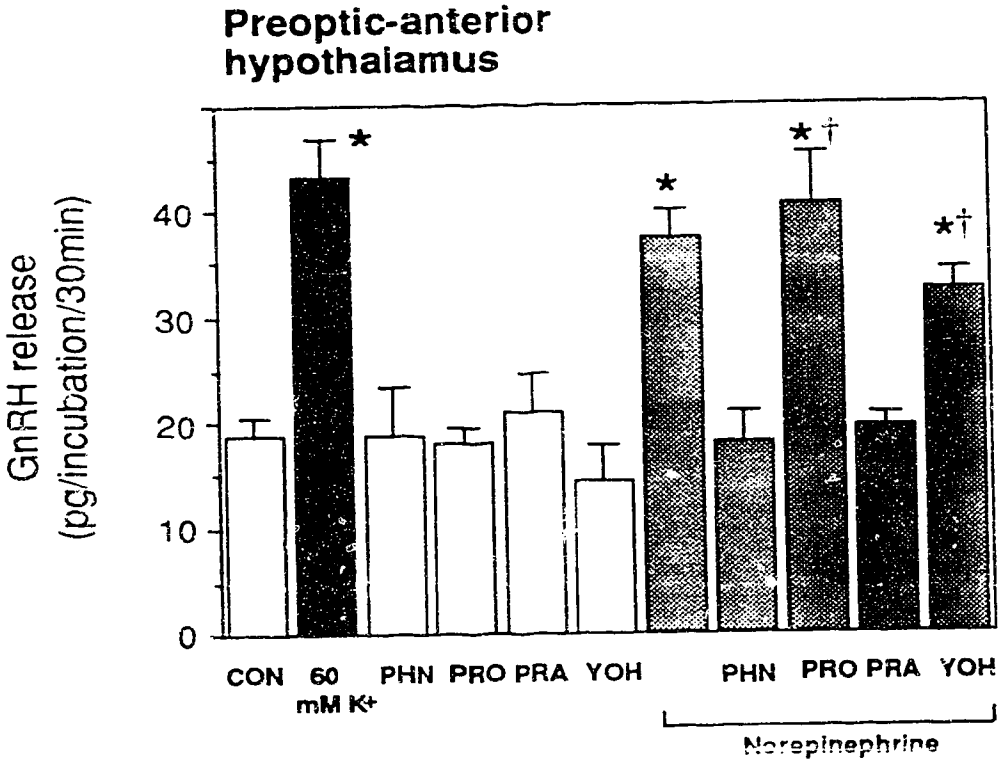

Fig. 7.3. Effects of the adrenergic receptor antagonists phentolamine (PHN), propranolol (PRO), prazosin (PRA) and yohimbine (YOH; all at 1 μM) on the spontaneous and NE- (60 μM) induced release of GnRH from preoptic-anterior hypothalamic slices of sexually mature male goldfish *in vitro*. Each column represents mean ± SEM (n=8). * indicates a significant difference ($p < 0.05$, one-way ANOVA and Duncan's multiple range test) from the control group. † indicates a significant difference ($p < 0.05$, Student's t-test) between corresponding antagonist-treated groups.

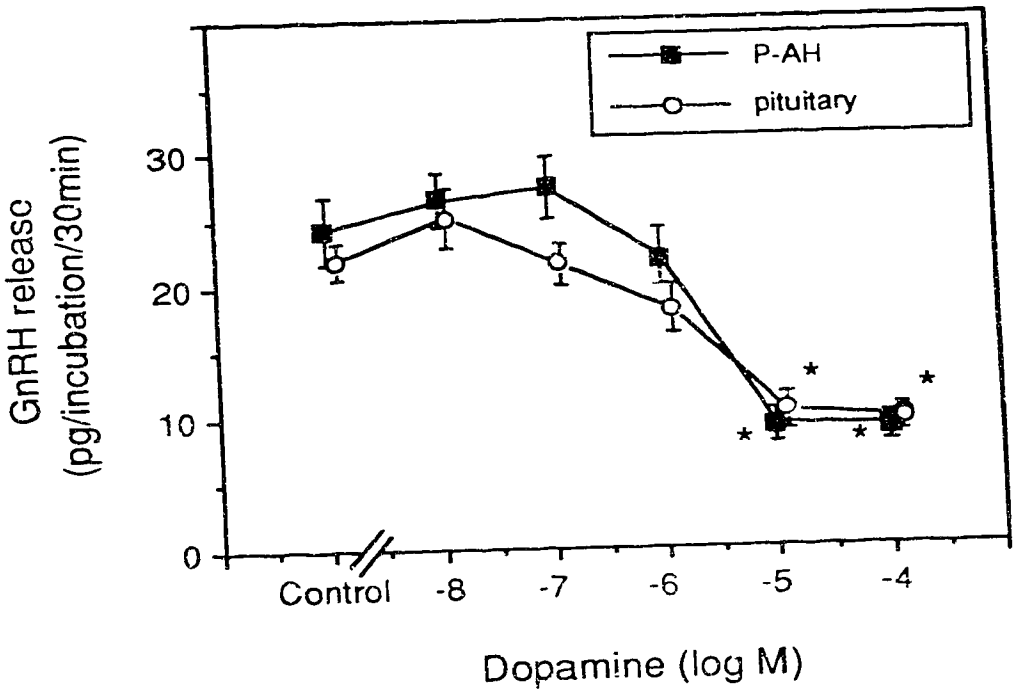

Fig. 7.4. Dose-response relationship between DA concentrations and GnRH release from preoptic-anterior hypothalamic (P-AH) slices and pituitary fragments of sexually mature male goldfish *in vitro*. Values represents mean ± SEM (n=8). * indicates a significant difference ($p < 0.05$, one-way ANOVA and Duncan's multiple range test) from the control group.

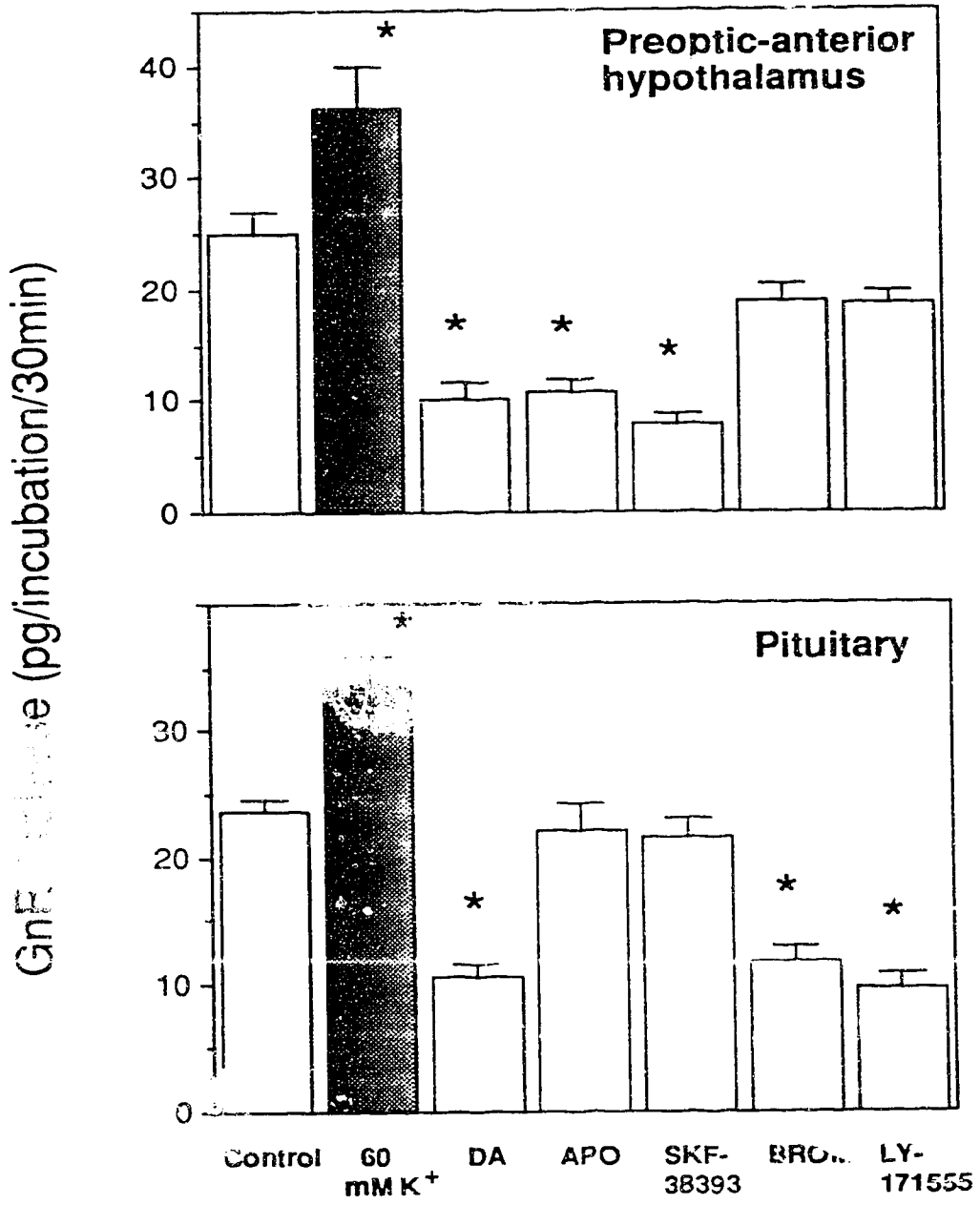

Fig. 7.5. Effects of DA (10 μM) and the dopaminergic receptor agonists apomorphine (APO), SKF-38393, bromocriptine (BROM) and LY-171555 (all at 1 μM) on release of GnRH from preoptic-anterior hypothalamic slices and pituitary fragments of sexually mature male goldfish *in vitro*. Each column represents mean ± SEM (n=8). * indicates a significant difference ($p < 0.05$, one-way ANOVA and Duncan's multiple range test) from the control group.

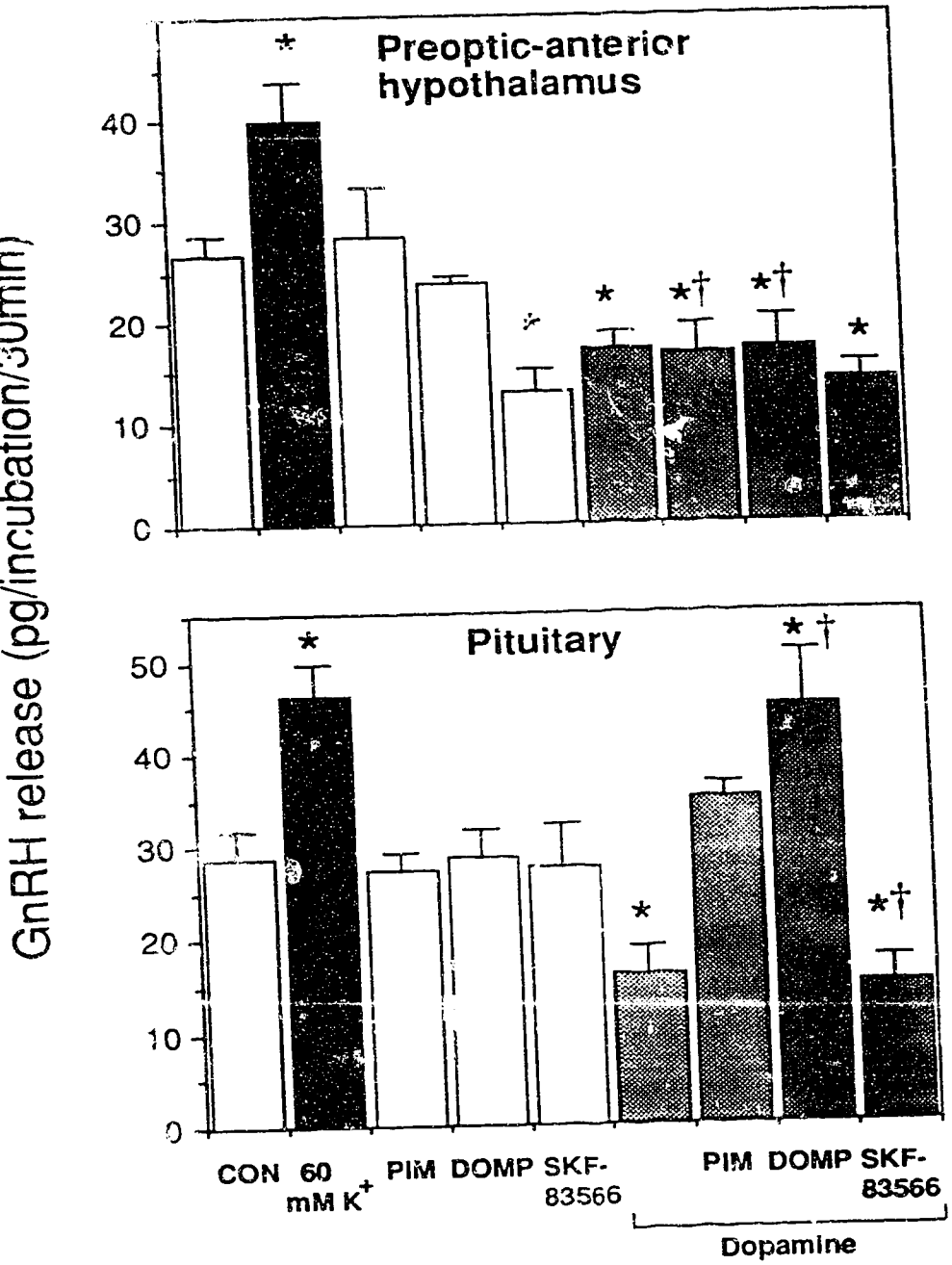

Fig. 7.6. Effects of the dopaminergic receptor antagonists pimozide (PIM), domperidone (DOM) and SKF-83566 (all at 1 μM) on the spontaneous and DA- (10 μM) induced inhibition of GnRH release from preoptic-anterior hypothalamic slices and pituitary fragments of sexually mature male goldfish *in vitro*. Each column represents mean + SEM (n=8). * indicates a significant difference ($p < 0.05$, one-way ANOVA and Duncan's multiple range test) from the control group (CON). † indicates a significant difference ($p < 0.05$, Student's t-test) between corresponding antagonist-treated groups.

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8. DIFFERENTIAL DISTRIBUTION OF TWO MOLECULAR FORMS OF GONADOTROPIN-RELEASING HORMONE IN DISCRETE BRAIN AREAS OF GOLDFISH (*CARASSIUS AURATUS*). [1]

8.1 INTRODUCTION

The existence of multiple molecular gonadotropin-releasing hormone (GnRH) forms in the brain of a single species has been well demonstrated in different vertebrates including birds, reptiles, amphibians, teleosts, elasmobranchs, ratfish and lamprey (see Sherwood, 1986). In teleosts, at least two immunoreactive GnRH (ir-GnRH) forms have been demonstrated in extracts of brain; a major form of ir-GnRH has been shown to be chromatographically and immunologically identical to [Trp^7, Leu^8]-GnRH (salmon GnRH or sGnRH; Sherwood *et al.*, 1983, 1984; Sherwood and Harvey, 1984; King and Millar, 1985; Powell *et al.*, 1986b; Sherwood, 1986). Although the structures of other molecular forms of GnRH in extracts of t[illegible]n are not known, the presence of [Gln^8]-GnRH (chicken GnRH-I or cGnRH-I; *Tilapia sparrmahii*; King and Millar, 1985) and [His^5, Trp^7, Tyr^8]-GnRH (chicken GnRH-II or cGnRH-II; *Coris julis*; Powell *et al.*, 1986b) have been suggested on the basis of high pressure liquid chromatography (HPLC) and radioimmunoassay (RIA) studies. The distribution of different ir-GnRH forms in the teleost brain has not been investigated. The present study describes the differential distribution of two ir-GnRH forms in discrete brain areas of female and male goldfish using reverse phase HPLC and RIA with different antisera.

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[1] A version of this chapter has been published: Yu, K. L., Sherwood, N. M., and Peter, R. E. 1988. Peptides 9: 625-630.

8.2 MATERIALS AND METHODS

Tissues

Female and male goldfish (25 to 35 g body weight), in early stages of gonadal recrudescence were purchased from Grassyfork Fisheries Company, Martinsville, IN. The fish were held in a 1800-liter flow-through aquaria at 15°C on a simulated natural (Edmonton) photoperiod for one week. A hundred and twenty-five brains and pituitaries were removed from female and male fish. All except 25 whole brains and pituitaries from fish of both sex, were rapidly dissected into different brain areas as described in Chapter 2 (Section 2.2, Materials and Methods) and rapidly frozen on dry ice.

Extraction

The frozen whole brains (from 25 female fish), dissected brain areas and pituitaries (100 equivalents from both sexes) were separately extracted for GnRH immunoactivities. The frozen tissues were powdered with liquid nitrogen, extracted in acetone: 1N HCL (100:3 v/v; 5 ml per 1 g tissue or 10 ml for tissues weighing less than 2 g), re-extracted in acetone: 0.01 N HCl (80:20 v/v) and defatted with petroleum ether (b.p. 30-60°C) as previously described (Sherwood *et al.*, 1986). The volume of the final aqueous phase was reduced in a refrigerated vacuum concentrator (SAVANT) to less than 1 ml. The soluble phase was filtered through a 0.22 µm Millipore GV filter and the volume adjusted to 1 ml with 'ORGANICpure' water (Barnstead NANOpure cartridge system).

High Pressure Liquid Chromatography

Chromatography of goldfish brain, spinal cord, and pituitary tissue extracts was performed on a Varian 5000 liquid chromatograph system. The tissue extract filtrate (800 µl) was injected via a 1-ml injection loop into a Supelco C-18 column (0.46 x 25 cm) with a

C_{18} guard column (Supelco, Inc., Bellefonte, PA). The mobile phase was acetonitrile (CH_3CN) in 0.25 M formic acid adjusted with triethylamine to pH 6.5 (TEAF), starting with an isocratic initial elution of 17% CH_3CN for 10 min., increasing during the following 7 min. to 24% CH_3CN, and staying at 24% CH_3CN for 33 min. The flow rate was 1 ml per min. The column outflow was collected in 1 ml fractions and aliquots assayed for immunoreactive GnRH using five antisera (B-6, b[illegible] 4, PB-45 and PBL-49).

Each injection of tissue extract was preceded by a blank run in which TEAF (800 µl) was injected and 1 ml fractions collected under the same conditions as for the tissue extracts. Fractions from the blank run were assayed for immunoreactive GnRH to determine whether there was any carry over of GnRH from one run to another. Fresh standards were chromatographed between sample runs for comparison with the brain tissue extracts. Synthetic mammalian GnRH (mGnRH), sGnRH, cGnRH-I, cGnRH-II were obtained from Peninsula Laboratories (San Carlos, CA), and [Tyr^3, Leu^5, Glu^6, Trp^7, Lys^8]-GnRH (lamprey GnRH) was a gift from Dan Marshak. The synthetic peptides (200 ng or 400 ng) were mixed together, diluted with TEAF to a final volume of 800 µl, and injected onto the HPLC column under the same conditions used for brain tissue extracts. Recovery of synthetic sGnRH and cGnRH-II after chromatography was estimated to be 88.6 ± 8.7% (n=4) and 79.6 ± 7.9% (n=4) respectively using antiserum PBL-49 in a sGnRH RIA (see Chapter 2) using sGnRH as iodinated tracer and sGnRH and cGnRH-II as respective standards.

Radioimmunoassay

GnRH immunoactivity in the HPLC fractions of extracts from whole brain and different brain parts were measured using a mGnRH RIA with synthetic mGnRH as iodinated tracer and sGnRH as standards (Sherwood *et al.*, 1983; 1986) and a sGnRH

RIA with synthetic sGnRH as iodinated tracer and standards (see Materials and Methods, Chapter 2). Five antisera with different specificity towards various known vertebrate GnRH peptides were used: GF-4 and PBL-49 (gift from W. W. Vale) were produced against sGnRH; B-6 against mGnRH; PB-45 (gift from W. W. Vale) against [lys^8]-GnRH and Bla-4 was generated against lamprey GnRH. The specificity of the antisera in the two RIA systems is shown in Table 8.1.

The immunoreactive materials from the major peak fractions were assayed in serial doubling dilutions using the mGnRH RIA with antiserum GF-4 and the sGnRH RIA with antiserum PBL-49. Linear displacement curves produced by synthetic GnRH peptides and the HPLC fractions were constructed using logit-log transformation. Parallelism between the various displacement curves was determined by using analysis of covariance or by comparing the slope of the two regression lines using t-test (Snedecor and Cochran, 1976).

8.3 RESULTS

Reverse phase HPLC of extracts of whole brain, various brain areas, spinal cord and pituitary from female goldfish, and RIA with two antisera (GF-4 and PBL-49; data with antiserum PBL-49 not shown) revealed two peaks of GnRH immunoactivity (Figs. 8.1, 8.2). The two immunoreactive peaks coeluted with synthetic cGnRH-II and sGnRH. Antisera Bla-4 and B-6 revealed no immunoreactive peaks coeluting with synthetic lamprey GnRH and mGnRH respectively. No sexual differences in the molecular forms of GnRH immunoactivity in brain extracts were detected (data not shown).

To compare the immunoactivity of ir-GnRH materials in the two peaks, serial dilutions of the early eluting and the late eluting immunoactivity were assayed using the sGnRH RIA with antiserum PBL-49; this RIA was previously shown to distinguish between sGnRH and cGnRH-II on the basis of differences in the slope of the displacement curves (in Chapter 2). The GnRH immunoactivity in the late eluting peak from extracts of

various brain areas yielded serial dilution displacement curves parallel to those of synthetic sGnRH (Fig. 8.3, upper panel). By contrast, the displacement curves of the ir-GnRH material in the early eluting peak from extracts of each brain area were not parallel to sGnRH ($p<0.01$, t-test); however, the displacement curves for the extracts were parallel to synthetic cGnRH-II in this RIA (Fig. 8.3, lower panel). Together with the HPLC analysis, this similarity in immunoactivity suggests a possible similarity in structure. In the mGnRH RIA using antiserum GF-4, the displacement curves of early eluting and the late eluting ir-GnRH material were parallel to both synthetic cGnRH-II and sGnRH, respectively (data not shown).

Table 8.2 shows the levels of the early and late eluting GnRH immunoactivity (using cGnRH-II and sGnRH as standards respectively) in the same HPLC fraction of the whole goldfish brain extract as measured by different radioimmunoassays using different antisera. Estimates of the sGnRH content in the late eluting peak were similar using each of the three antisera (GF-4, PB-45 and PBL-49), indicating that the immunoactivity of the ir-GnRH material in the late eluting peak is similar to the synthetic sGnRH molecule. However, there was variation in the estimates of cGnRH-II content in the early eluting peak. This may reflect the poor crossreactivity of the antisera used in this study to cGnRH-II (Table 8.1).

The distribution of cGnRH-II-like immunoactivity (cGnRH-II-LI) and sGnRH-like immunoactivity (sGnRH-LI) in the early and late eluting peaks respectively, as measured by the sGnRH RIA with antiserum PBL-49, in eight subdivisions of the female and male goldfish brain is illustrated in Fig. 8.4. Both molecular forms of GnRH show a wide distribution in the goldfish brain. For sGnRH-LI, it is more concentrated in the pituitary, telencephalon and hypothalamus than in other brain areas. On the other hand, cGnRH-II-LI showed a more even distribution in both female and male brain. The concentrations of both ir-GnRH forms in various brain areas are similar in both sexes, although

cGnRH-II-LI has a lower concentration in the pituitary and spinal cord in male goldfish compared to the females. Notably, the proportion of the early eluting cGnRH-II-LI to the late eluting sGnRH-LI is generally higher in the caudal parts (optic tectum-thalamus, cerebellum, medulla and spinal cord) compared to the rostral parts (olfactory bulbs, telencephalon, hypothalamus and pituitary) of the brain. This differential distribution pattern of cGnRH-II-LI and sGnRH-LI in various parts of brain is similar in both female and male goldfish (Fig. 8.4). Similar results were obtained using the mGnRH RIA with antiserum GF-4 (data not shown). Also, cGnRH-II-LI and sGnRH-LI are found in all brain areas using antisera (Bla-4, GF-4, PB-45 and PBL-49) which crossreact with synthetic cGnRH-II and sGnRH, although the absolute numbers were somewhat different for the amount of cGnRH-II-LI present.

8.4 DISCUSSION

The present study confirmed the presence of sGnRH and demonstrated that the second major form of GnRH present has similar chromatographic and immunological properties as cGnRH-II in the brain, spinal cord and pituitary of male and female goldfish. Two or more forms of GnRH have previously been described in teleost brain. All teleost species investigated to date have sGnRH (Sherwood *et al.*, 1983, 1984; Sherwood and Harvey, 1984; King and Millar, 1985; Powell *et al.*, 1986b; Sherwood, 1986). It was reported that the 'early eluting peak of trout GnRH-like peptide' or 'salmon GnRH II' in striped mullet, milkfish, rainbow trout and salmon, eluted in the same position as synthetic cGnRH-II in a HPLC system (Sherwood and Harvey, 1984; Sherwood *et al.*, 1986). This 'salmon GnRH II' was also found in brain of frogs, salamander (see Sherwood *et al.*, 1986), dogfish shark, ratfish and herring (see Sherwood, 1986) in HPLC studies. Using different HPLC systems, cGnRH-II was also shown to be present in brain extracts

of two reptiles (an alligator and skink, Powell *et al.*, 1986a), toad (King and Millar, 1986) and a teleost (*Coris julis*, Powell *et al.*, 1986b). If the early eluting ir-GnRH form in goldfish and the 'salmon GnRH II' in various vertebrate species are structurally identical to cGnRH-II, as the present results suggest, cGnRH-II would represent the most widespread known GnRH form in vertebrates (see Sherwood, 1986).

Chromatographic evidence confirms RIA data that there is no significant mGnRH and lamprey GnRH forms in the goldfish brain. Antiserum B-6 which crossreacts with mGnRH, but not other known vertebrate GnRH forms, showed no crossreactivity with goldfish brain extract, or the HPLC fraction coeluting with synthetic mGnRH. Since antiserum B-6 also can recognize GnRH-like molecules in sturgeon (see Sherwood, 1986), this indicates that the sturgeon GnRH-like molecules are different from the two forms described in goldfish brain. Similarly, antiserum Bla-4 which recognizes lamprey GnRH, showed no crossreactivity with the HPLC fractions coeluting with lamprey GnRH.

This study demonstrated that the two GnRH forms are expressed in all brain areas, spinal cord and pituitary of female and male goldfish. The distribution of the late eluting sGnRH-LI in various brain regions of goldfish generally agrees with the immunocytochemical studies (Kah *et al.*, 1984, 1986) showing that the ir-GnRH cell bodies are mainly located in the rostral forebrain regions. On the other hand, cGnRH-II-LI showed a more even distribution over the goldfish brain including optic tectum- thalamus, cerebellum and medulla regions, where no or few GnRH immunoreactive cell bodies have been described previously (Kah *et al.*, 1984, 1986). These data suggest that the cGnRH-II-like immunoreactive structures in the goldfish brain are anatomically separated from the sGnRH-like immunoreactive neurons and fibers described previously. Since the available GnRH antisera showed a relatively low crossreactivity toward cGnRH-II, it is quite possible that cGnRH-II immunoreactive cell bodies were poorly immunostained and not identified in previous studies on teleost brain. Development of specific antisera towards

cGnRH-II is needed for the immunocytochemical localization of cGnRH-II-like immunoreactive GnRH structures in the goldfish brain.

The ir-GnRH structures in the vertebrate olfactory system (Peter, 1986) have been hypothesized to have an important function in reproduction in mammals (Witkin and Silverman, 1983) and in teleosts (Demski and Northcutt, 1983). However, the nature of the GnRH demonstrated in olfactory systems by immunocytochemistry has not previously been investigated. This study revealed that the goldfish olfactory system contains both sGnRH-LI and cGnRH-II-LI as in the other parts of the brain and pituitary.

Quantitation of cGnRH-II-LI and sGnRH-LI in the early and late eluting peaks respectively showed that the distribution of the two ir-GnRH forms in the eight subdivisions of the goldfish brain is different. The proportion of the early eluting cGnRH-II-LI to the late eluting sGnRH-LI is generally higher in the caudal parts (optic tectum-thalamus, cerebellum, medulla and spinal cord) compared to the rostral parts (olfactory bulbs, telencephalon, hypothalamus and pituitary) of the brain. Similar differential distribution of the two ir-GnRH forms was obtained in both female and male goldfish brain using two RIA with two different antisera.

This is the first study in teleosts to show differential distribution of two GnRH forms in the brain. Using two antisera raised against cGnRH-I and cGnRH-II respectively, the amount of the two chicken GnRH forms was measured separately in 6 subdivisions of the male chicken brain by RIA (Hasegawa *et al.*, 1986). The results showed that the caudal brain (cerebellum and medulla oblongata) of the male chicken had a much higher content (4 and 11 fold higher respectively) of cGnRH-II immunoactivity than cGnRH-I, whereas the hypothalamus and preoptic region contained mainly cGnRH-I immunoactivity (10 fold higher than that of cGnRH-II). Such differential distribution of two GnRH forms in birds was confirmed by immunocytochemical studies in chicken and Japanese quail brains (in Hattori *et al.*, 1986), showing that cGnRH-I immunoreactive cell bodies were found in the nucleus preopticus and the nucleus septalis, and cGnRH-I

immunoreactive fibers were found in the median eminence; on the other hand, cGnRH-II immunoreactive fibers were not detected in the median eminence, and cGnRH-II immunoreactive cell bodies were detected mainly in midbrain, specifically the lateral parts of the nucleus tuberalis and in the region anterior to dorsal part of the root of nervus oculomotorius. The pituitary gland of teleosts is directly innervated by neurosecretory fibers, the homologue of the tetrapod median eminence (see Schreibman, 1986); unlike the immunocytochemical studies on chicken and Japanese quail, both forms of GnRH were detected in the pituitary of goldfish using HPLC and RIA. Nevertheless, it is apparent that there is a differential distribution of two forms of GnRH in both birds (chicken and Japanese quail) and teleosts (goldfish).

The existence of two structurally similar peptides in various parts of the brain of a single species is interesting from the functional point of view. Using a sGnRH RIA with antiserum PBL-49, changes in the concentrations of sGnRH have been found in the olfactory bulbs, telencephalon, hypothalamus and pituitary during the periovulatory period in female goldfish (Chapter 2). This study showed that the total sGnRH-LI in the rostral parts of the goldfish brain is physiologically labile during the periovulatory period. Since ir-GnRH fibers directly innervate pituitary cells in goldfish (Kah *et al.*, 1984, 1986), the presence of both GnRH forms in the pituitary suggests a role for both GnRH forms in the regulation of pituitary functions. It has been demonstrated that sGnRH and mGnRH have similar activity *in vivo* in goldfish (Peter *et al.*, 1985); likewise, mGnRH and cGnRH-II also have similar activity *in vivo* in goldfish (Peter *et al.*, 1987). The possibility that different forms of GnRH may have differential effects on various pituitary functions requires further investigation.

Although both forms of GnRH present in the goldfish may have a neuroendocrine function, the predominance of cGnRH-II-LI in the caudal brain suggests a different function for the peptide in this brain region. A variety of evidence suggests that GnRH

might play a neuromodulator or neurotransmitter role in the central nervous system: (i) wide distribution of GnRH in the brain of vertebrates (see Peter, 1986); (ii) the termination of GnRH axons on the caudal neurosecretory neurons in the spinal cord of a teleost (Miller and Kriebel, 1986); (iii) changes in sexual behavior following central injection of GnRH into the brain of vertebrates (Cheng, 1977; Alderete *et al.*, 1980; Moore *et al.*, 1982; Moss and Dudley, 1984); and, (iv) the presence of GnRH receptors in several brain regions in mammals (Millan *et al.*, 1986; Badr and Pelletier, 1987; Haour *et al.*, 1987). However, the role of different GnRH forms in modulating function in the central nervous system remains open for future study.

TABLE 8.1
Crossreactivity of Antisera to Various Vertebrate GnRH Peptides in Radioimmunoassays.

Antiserum	Iodinated Tracer	GnRH Standard	Percentage Crossreactivity * (%)				
			sGnRH	mGnRH	cGnRH-I	cGnRH-II	Lamprey GnRH
Bla-4	mGnRH	lamprey GnRH	15.0	12.5	50.0	38.0	100.0
B-6	mGnRH	mGnRH	0.02	100.0	0.02	0.02	0.02
PB-45	sGnRH	sGnRH	100.0	100.0	133.3	25.9	24.0
PBL-49	sGnRH	sGnRH	100.0	93.3	333.3	32.6	2.0
PBL-49	mGnRH	sGnRH	100.0	100.0	n.d.†	20.0	n.d.†
GF-4	mGnRH	mGnRH	44.0	100.0	25.0	6.3	0.02

* Percentage crossreactivity was measured at $B/Bo = 50\%$.

† n.d., not determined.

TABLE 8.2
Comparison of the Early Eluting cGnRH-II-LI and the Late Eluting sGnRH-LI in Extracts of Female Goldfish Whole Brain (n = 25) Using Four Different RIA.

Antiserum	Iondinated Tracer	Late Eluting sGnRH-LI (ng sGnRH standard)	Early Eluting cGnRH-II-LI (ng cGnRH-II standard)
GF-4	mGnRH	22.82	29.08
PB-45	sGnRH	34.77	26.03
PBL-49	sGnRH	23.33	70.98
PBL-49	mGnRH	25.90	41.25

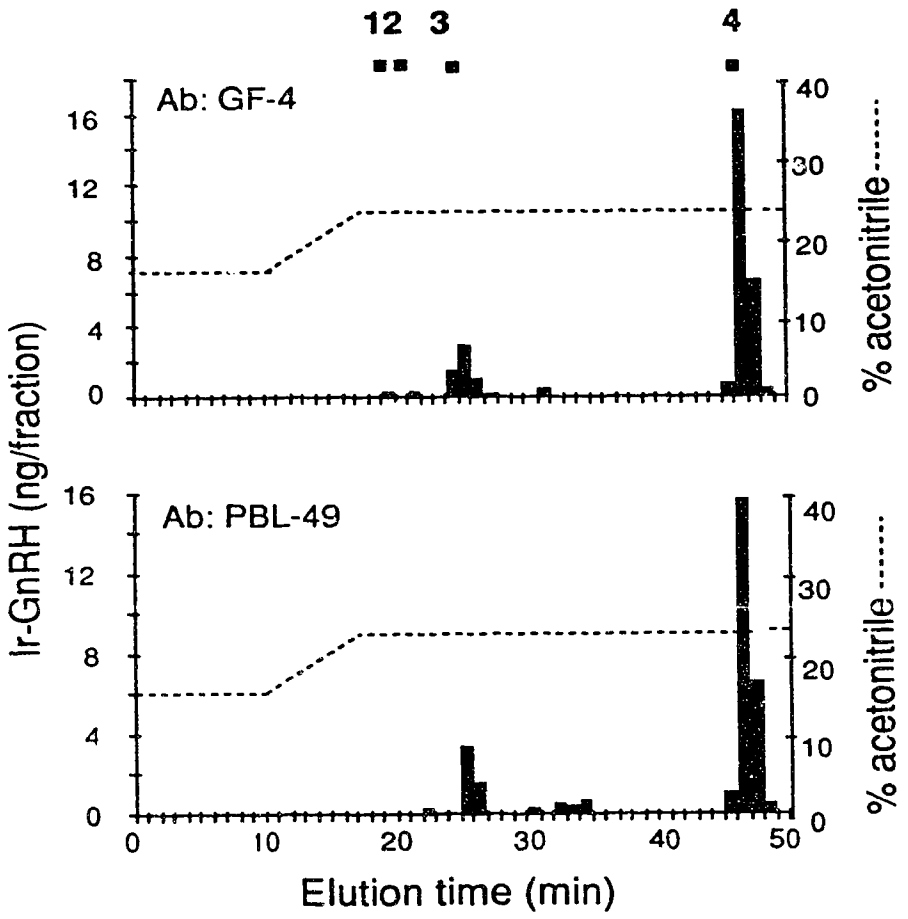

Fig. 8.1. Reverse phase HPLC of extracts of female goldfish whole brain (n=25) and RIA with two antisera (GF-4 and PBL-49). Elution positions of mGnRH and lamprey GnRH (1), cGnRH-I (2), cGnRH-II (3) and sGnRH (4) are indicated. Each column represents the total amount (mean of duplicate determinations) of ir-sGnRH in each 1 ml fraction (flow rate of 1 ml/min). The mobile phase was acetonitrile in TEAF, at pH 6.5. Percent acetonitrile is shown as a dotted line. Aliquots (50 ml) of the HPLC fractions were assayed with antiserum GF-4 (upper panel) in a mGnRH RIA (mGnRH as iodinated tracer and sGnRH as standards) and with antiserum PBL-49 (lower panel) in a sGnRH RIA (sGnRH as iodinated tracer and sGnRH as standards).

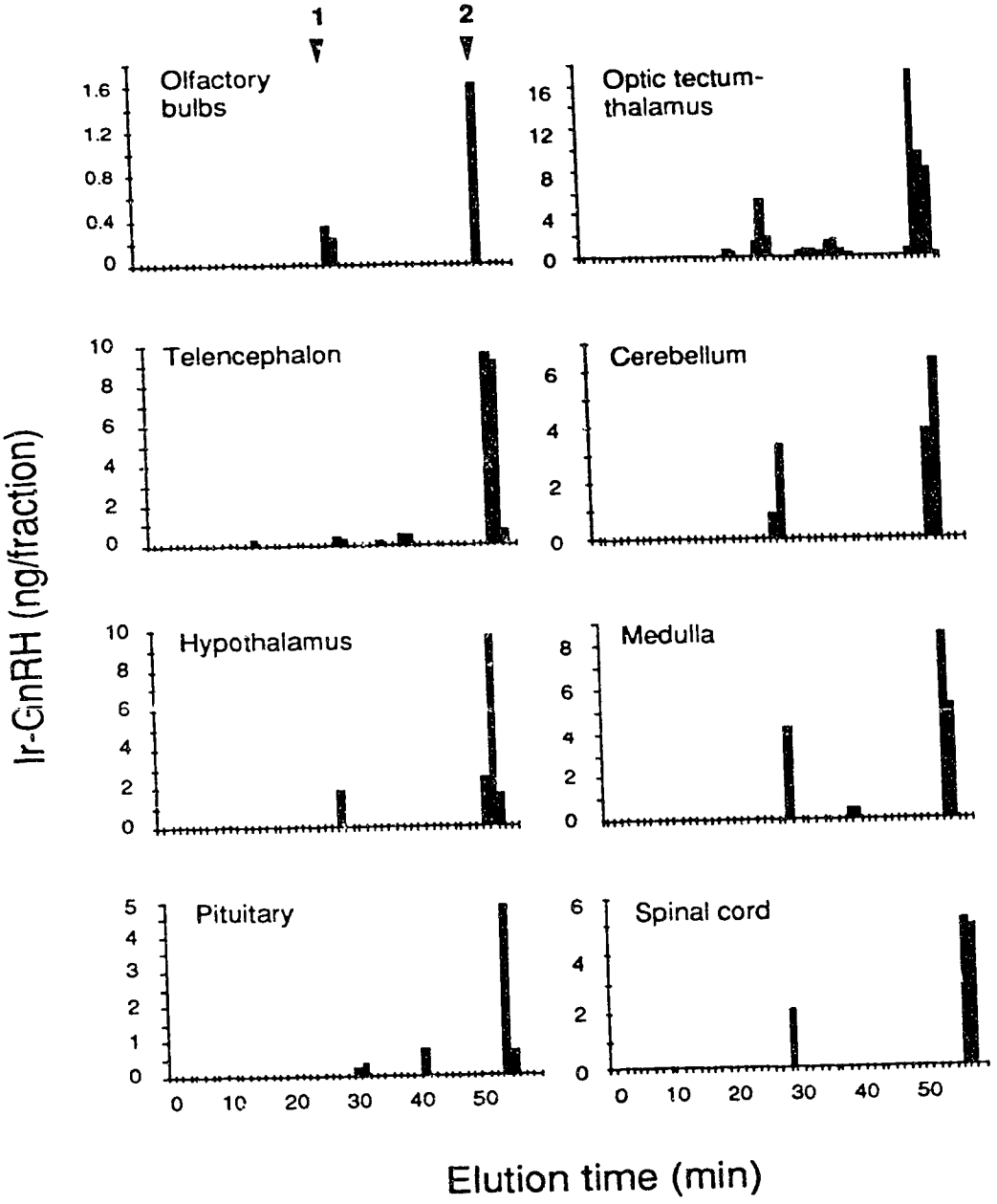

Fig. 8.2. Reverse phase HPLC of extracts of various brain areas, spinal cord and pituitary from female goldfish (n=100). Fractions were assayed with antiserum GF-4 using a mGnRH RIA with synthetic mGnRH as iodinated tracer and sGnRH as standards. Elution positions of cGnRH-II (1) and sGnRH (2) are indicated. Each column represents the total amount (mean of duplicate determinations) of ir-GnRH in each 1 ml HPLC fraction (flow rate of 1ml/min).

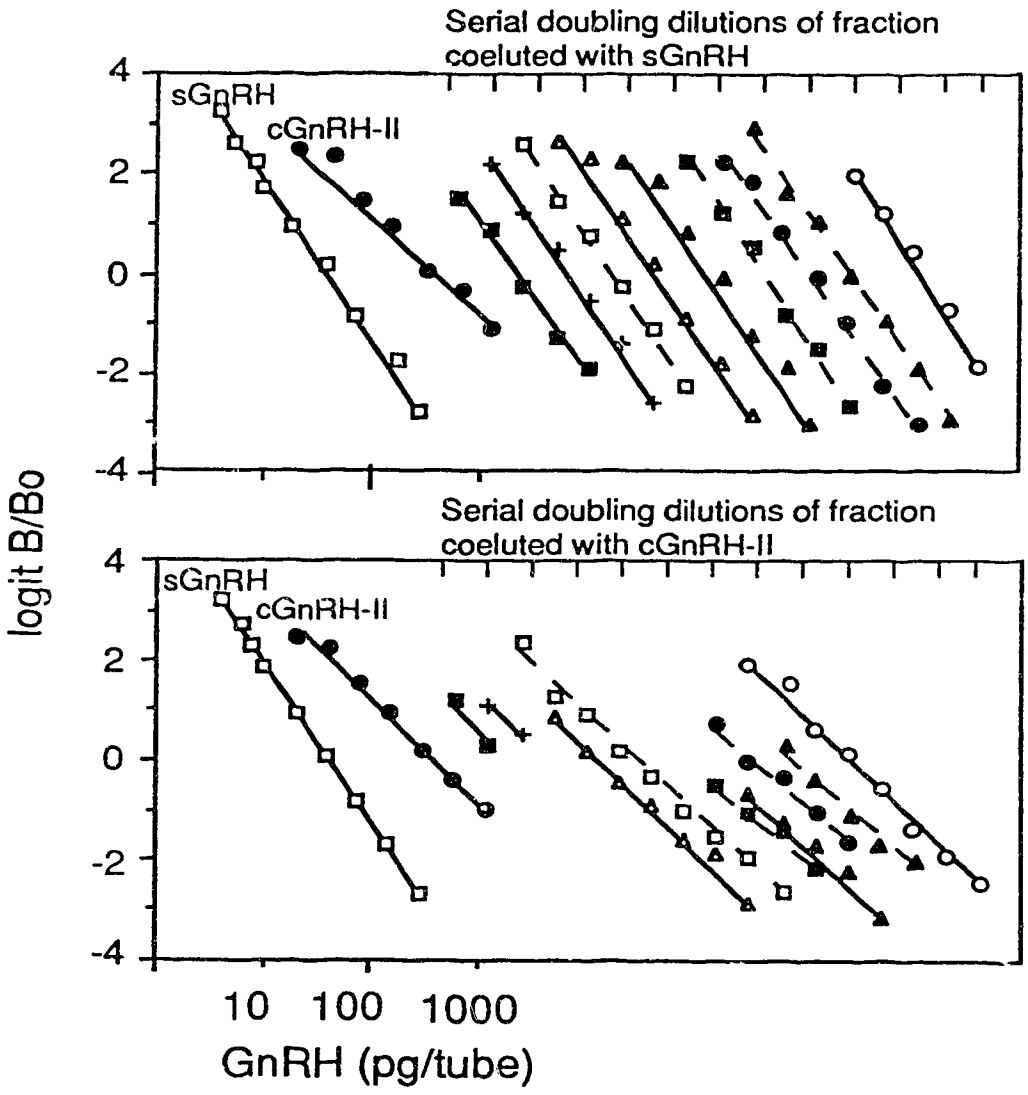

Fig. 8.3. Comparative displacement of ^{125}I-sGnRH from the antiserum PBL-49 by synthetic sGnRH, cGnRH-II, the late eluting (fraction 47, upper panel) and the early eluting (fraction 26, lower panel) immunoreactive material in extracts of various brain areas (—■— olfactory bulbs; —+— pituitary; —□— optic tectum-thalamus; —△— medulla; —▲— spinal cord;---■--- hypothalamus; ---●--- telencephalon; ---▲--- cerebellum; —○— whole brain). Each point represents the mean of duplicate determinations.

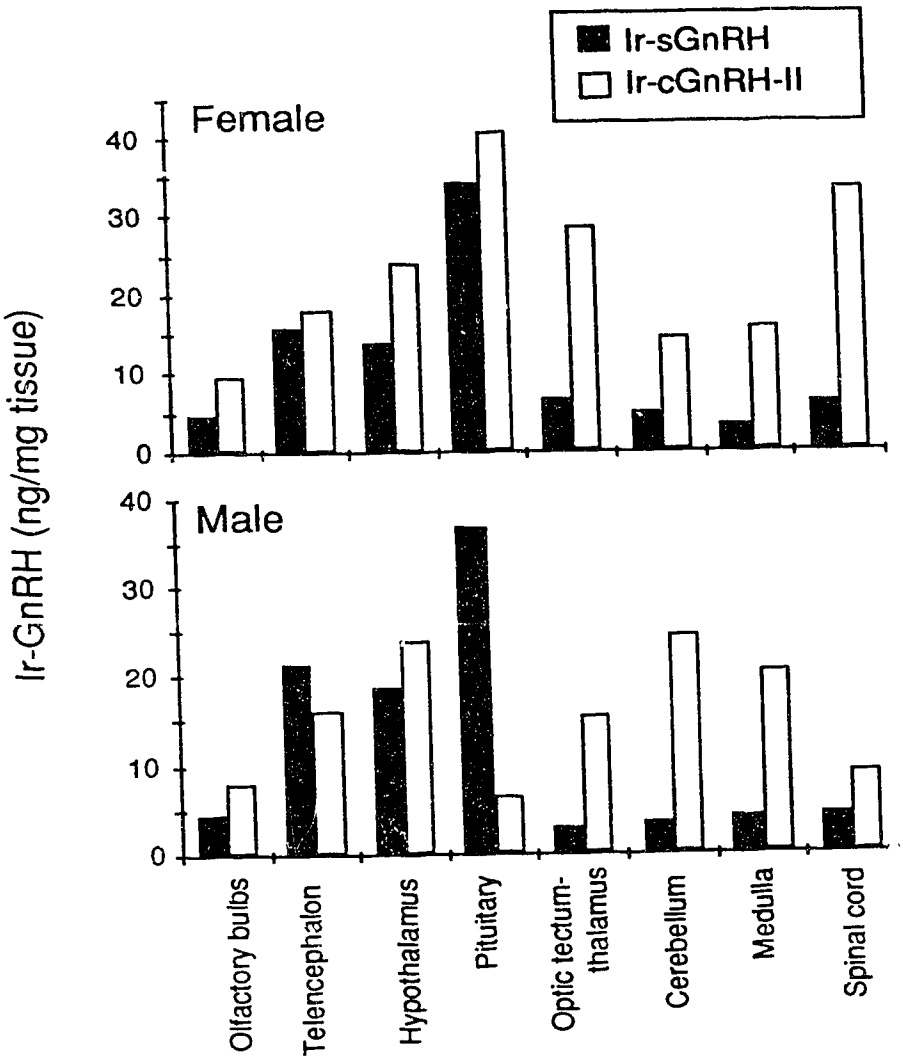

Fig. 8.4. Quantitation of cGnRH-II-LI and sGnRH-LI in the early and late eluting peaks respectively by a sGnRH RIA with antiserum PBL-49 using iodinated sGnRH as tracer and cGnRH-II and sGnRH as respective standards in eight subdivisions of the female (upper panel) and male (lower panel) goldfish brains (n=100). Concentrations of ir-GnRH were calculated from wet tissue weight. Each column represents mean of duplicate determinations.

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GnRH immunoactivity present in the void volume following chromatography of goldfish brain extracts on a gel filtration column, indicating the lack of crossreactivity with larger precursor molecules. Finally, the molecular heterogeneity of the immunoreactive GnRH (ir-GnRH) was studied by high performance liquid chromatography (HPLC) as described in Chapter 8. Results showed that sGnRH is the major form of GnRH responsible for the immunoactivities detected in the pituitary and various brain areas using this RIA; a second GnRH form recognized by this RIA was characterized to be [His^5, Trp^7, Tyr^8]-GnRH (chicken GnRH-II or cGnRH-II) by HPLC and RIA analysis (Chapter 8). The performance characteristics (precision and sensitivity) of the RIA make possible detection of picogram quantities of GnRH decapeptide in extracts from pituitary and discrete brain areas of a single goldfish (20-35 g).

Using this RIA, radioimmunoassayable GnRH was found in all brain areas and the pituitary in goldfish, with the highest concentrations in the pituitary; GnRH concentrations in the olfactory bulbs, telencephalon and hypothalamus are higher than those in the optic-tectum thalamus region, cerebellum and spinal cord (Chapter 2). Results from immunocytochemical studies (Kah *et al,* 1986) further confirms the validity of the sGnRH antiserum (PBL-49) and extends the results obtained by the RIA (see above). Ir-GnRH fibers were found to be widely distributed in the goldfish brain with cell bodies located in an apparent continuum starting anteriorly from the olfactory bulbs, along the ventral telencephalon and concentrated in the preoptic region (Kah *et al,* 1986) similar to other vertebrates (see Peter, 1986). Direct innervation of the pars distalis of the pituitary by ir-GnRH fibers was also demonstrated in goldfish (Kah *et al,* 1986), providing the anatomical substrate for GnRH regulation of pituitary GtH secretion.

Studies in Chapter 2 showed that regression of the ovary in goldfish is not associated with depletion of GnRH in the pituitary and brain. Clear seasonal changes in brain GnRH levels parallel to seasonal ovarian recrudescence were not observed. The

involvement of the central GnRH neuronal systems in ovarian recrudescence requires further investigation.

While many studies have concerned the neuroendocrine control of pituitary GtH secretion in fishes (see Kah, 1986; Peter *et al*, 1986; de Leeuw, 1987), few studies contribute directly to our understanding of the regulation of brain GnRH neuronal activity in teleosts. The present study is the first to describe the temporal and region specific changes in GnRH concentrations in discrete brain areas and the pituitary in association with serum GtH changes during the periovulatory period in a teleost (Chapter 2 and 5). Decreases in GnRH levels were first observed in the olfactory bulbs, telencephalon and hypothalamus (and sometimes in pituitary, Chapter 5) at approximately 17 hours prior to ovulation and remained low during ovulatory GtH surges; GnRH levels in pituitary decreased at the time of the ovulatory surge of GtH. Similar decreases in GnRH levels in discrete hypothalamic nuclei and the median eminence (ME) associated with the LH surge on the afternoon of proestrus have also been observed in rats (Rance *et al*, 1981; Wise *et al*, 1981). In short-tailed field voles, the copulation induced LH surge and reflex ovulation were also associated with a marked drop in hypothalamic GnRH levels (Versi *et al*, 1982). Other studies showed that preovulatory surges in hypophyseal portal blood levels of GnRH occurred during reflex ovulation in rabbits (Tsou *et al*, 1977), and during spontaneous ovulation in rats, as well as in other mammals (see Fink, 1988; Lincoln, 1988). These studies showed that temporal specific changes in GnRH levels in localized discrete brain areas during the periovulatory period, at least in some mammalian species, could be used as an index of GnRH neuronal activity despite the fact that tissue levels of GnRH reflect a balance of the biosynthesis, transport, release and degradation of the peptide. On this basis, the negative correlation between brain GnRH and serum GtH levels observed during ovulation in goldfish in this study supports the view that the ovulatory surge of GtH is due to hypersecretion of GnRH in teleosts (see Peter *et al.*, 1986).

Furthermore, the concomitant decreases in GnRH concentrations in different forebrain areas and the pituitary suggests that various components of the GnRH neuronal system may function as an integrated unit, and are activated during spontaneous ovulation in goldfish. This hypothesis emphasizes the involvement of different GnRH neurons in various brain areas in the neural circuitry for the regulation of pituitary GtH secretion, and the existence of an interrelationship between GnRH neurons. In contrast, it was generally thought that the preoptic-hypophysial GnRH pathway mediates the GnRH regulation of pituitary GtH secretion in teleosts although direct evidence is lacking (see Ball, 1981; Peter, 1986). Immunocytochemical studies combined with brain lesions in goldfish showed that ir-GnRH fibers in the pars distalis of the pituitary originate primarily from the cell bodies in the preoptic region (Kah *et al*, 1986). However, studies in goldfish involving lesions of the preoptic region result in stimulation of pituitary GtH secretion, presumably because of the damage of the gonadotropin release-inhibitory factor (GRIF) in the same region; substantial evidence suggests that dopamine (DA) has GRIF activity in goldfish (see Peter *et al*, 1986). By demonstrating the alterations in GnRH levels in the brain areas (the telencephalon including preoptic region, and hypothalamus) and the pituitary along the preoptic-hypophysial GnRH pathway in goldfish during the ovulatory GtH surge (Chapter 2 and 5), the present study confirms an important role of the preoptic GnRH neurons in the regulation of pituitary GtH secretion.

In addition, the present study provides, for the first time, physiological evidence to suggest that GnRH neurons in the olfactory bulb region are also involved in the GtH surges during spawning in female (Chapters 2 and 5) and male goldfish (Chapter 5) by demonstration of temporally specific changes in GnRH levels in this region. Since the GnRH neurons in the olfactory bulbs region belong to a part of the terminal nerve (TN) system, results of the present study suggest the activation of TN during ovulation in goldfish. It has been suggested that the TN mediates the behavioral or hormonal responses to environmental or pheromonal cues in teleosts (see Demski and Northcutt, 1983; Stell and

supports the previous suggestion that the GnRH neuronal system in goldfish may function as an integrated unit (see Chapter 2). The changes in GnRH levels in the olfactory bulb region in male goldfish during spawning behavior suggests that GnRH neurons in this region may be involved in the behavioral and hormonal response to PG-treated females. Since the GnRH neurons in the TN contained in the olfactory system have been shown to innervate the olfactory lamellae (Kyle and Stell, 1988), retina and forebrain areas (von Bartheld *et al.*, 1984; Stell and Walker, 1987), it is possible that the altered GnRH neuronal activity in the GnRH-containing TN during spawning behavior in male goldfish represents an integrated mechanism through which olfactory and visual components of the responses to sex pheromones are coordinated (Stell and Walker, 1987). This neurotransmitter or neuromodulator role of GnRH has been supported by the wide distribution of GnRH in the brain of goldfish, as well as in other vertebrates (see Discussion in Chapter 8). Nevertheless, the specific involvement of either the TN or olfactory nerve system in mediating the behavioral and hormonal responses to pheromonal stimuli in male goldfish has not yet been ascertained (see Kyle *et al*, 1987).

The lack of significant changes in the GnRH levels in the pituitary during spawning behavior of males with PG-treated females suggests that the GnRH in nerve terminals contained in the pituitary may not be causally related to the increases in serum GtH levels. However, the GnRH levels in the cell bodies contained in specific brain areas (olfactory bulbs, telencephalon) increased during spawning behavior with PG-treated females. These observations together suggest that exposure to PG-treated females likely has a stimulatory effect on the synthesis of GnRH in the brain through combined pheromonal and behavioral inputs to the GnRH neuronal system, without a concomitant major release of GnRH in the pituitary, resulting in a net increase in brain GnRH levels. This interpretation is consistent with results from studies in Chapter 4 and 5.

The olfactory tracts sectioning experiments described in Chapter 3 confirm results

of other studies showing the importance of medial olfactory tract pathways in mediating endocrine responses to sex pheromones (see Kyle *et al.*, 1987). It has been shown that the GtH response of male goldfish to PG-treated females depends on exposure to PG pheromones and subsequent behavioral interaction with conspecifics (Kyle *et al.*, 1983; Sorensen *et al.*, 1989). On this basis, the blockade of the increases in brain GnRH and serum GtH levels during spawning behavior with PG-treated females by olfactory tract sectioning in this study (in Chapter 3) is probably due to the absence of behavioral interaction, rather than the disruption of olfaction per se.

A major finding of the present study is the demonstration of a dopaminergic regulation of brain and pituitary GnRH levels in goldfish as described in Chapter 4. Pimozide, a centrally active DA receptor antagonist, caused a time and dose dependent increase in serum GtH levels, and accumulation of GnRH in the olfactory bulbs, telencephalon and pituitary. These effects of pimozide were partially reversed by the DA receptor agonist apomorphine which, when given alone, did not influence the basal levels of brain GnRH and serum GtH levels. These results suggest that the central dopaminergic system has a tonic inhibitory influence on brain and pituitary GnRH levels in goldfish. In view of the inhibitory actions of DA on GnRH secretion (see Chapters 6 and 7), the positive correlation between brain GnRH levels and serum GtH levels after pimozide treatment suggests that pimozide may stimulate synthesis and/or decrease in degradation relative to the rate of transport or release of GnRH peptides in the goldfish brain. This interpretation agrees with the observation that pimozide increases GnRH levels in areas of the goldfish brain containing GnRH cell bodies (olfactory bulbs, telencephalon; Kah *et al.*, 1986), and that DA stimulates degradation of GnRH in rat synaptosomes (Marcano de Cotte *et al*, 1980).

An important finding presented in Chapter 4 was the marked differences in changes in brain GnRH and serum GtH levels during spawning behavior with PG-treated females in normal and pimozide-treated male goldfish. The observation that pimozide-treated

goldfish, but not normal fish, have a marked reduction in brain and pituitary GnRH levels associated with a large increase in serum GtH levels in response to PG-treated females suggests that brain GnRH neurons are tonically inhibited by the central dopaminergic system. These results, together with results from other studies (see Peter *et al.*, 1986), suggest that the central dopaminergic system has a tonic inhibitory influence on pituitary GtH secretion by acting at the level of both pituitary gonadotrophs and brain GnRH neurons. Therefore, by blocking the central dopaminergic inhibition on the GnRH neurons, a large pool of GnRH peptides becomes available for the stimulation of pituitary GtH secretion upon receiving the stimuli from PG-treated females. The activation of pituitary GtH secretion by both augmented release of GnRH peptides (reflected by the decrease in pituitary GnRH levels) and removal of the inhibitory effects of DA directly on gonadotrophs (see Peter *et al*, 1986) results in the marked increase in serum GtH levels in pimozide-pretreated male goldfish during spawning behavior with PG-treated females.

The increases in brain GnRH and serum GtH levels during spawning behavior can be blocked by pretreatment with a DA receptor agonist apomorphine, which does not alter the behavioral responses to PG-treated females (Chapter 4). The results support the previous hypothesis that the central dopaminergic system has an inhibitory influence on pituitary GtH secretion by acting at both pituitary and brain levels, and that its disinhibition is, at least in part, involved in the stimulatory effects of spawning behavior on the brain GnRH and serum GtH levels in male goldfish.

Successful reproduction involves synchronization of gonadal maturation and sexual behavior between males and females. In goldfish, a serum GtH surge occurs in the male (Kobayashi *et al,* 1986; Stacey *et al*, 1989) at a similar time as that in the female (Stacey *et al*, 1979) during spawning; however, little is known about the neuroendocrine mechanisms controlling the serum GtH surges in the males. Studies presented in Chapter 5 describe, for the first time, the temporal changes in brain levels of GnRH and serum levels

of GtH and growth hormone (GH) in male goldfish spawning with spontaneously ovulating females. In addition to the demonstration of a GtH surge in the spawning male goldfish as described previously (Kobayashi *et al*, 1986; Stacey *et al*, 1989), the present study also shows that serum GH levels significantly increase in the spawning males at the time of ovulation by accompanying females. This represents the first study in a teleost to describe simultaneous increases in both serum GtH and GH levels in the spawning males in synchrony with the ovulatory surge of serum GtH (Stacey *et al*, 1979) and GH (Marchant, 1983) in accompanying female goldfish.

In addition, the present study demonstrates that the increases in serum GtH and GH levels in spawning male goldfish are associated with marked reductions in GnRH levels in the olfactory bulbs, telencephalon, hypothalamus and pituitary. These reductions in brain and pituitary GnRH levels are analogous to those in ovulating females (see also Chapter 2) and in pimozide pretreated males undergoing spawning behavior with PG-treated females (Chapter 3). These results, together with the recent findings that GnRH is capable of stimulating both GtH and GH release in goldfish (Marchant *et al.*, 1989; Marchant and Peter, 1989), suggest that activation of the GnRH neuronal system may be a common pathway for the stimulation of pituitary GtH and GH secretion in both male and female goldfish during spawning. Furthermore, if one considers the possibility raised in the present study that the reduction in GnRH levels in discrete forebrain areas and the pituitary may be responsible for the GtH surge during spawning in male and female goldfish, then, it is noteworthy that the rapid decline in serum GtH and GH levels is associated with the return of brain and pituitary GnRH levels to normal after spawning.

The simultaneous activation of the hypothalamo-hypophyseal-gonadal axis in both male and female goldfish during spawning appears to be mediated by two pheromones released in a sequential manner by ovulating females (see Sorensen and Stacey, 1989). In a series of studies, it has been shown that the serum GtH levels in male goldfish during

spawning are stimulated by two pheromones released in temporal sequence from ovulating females: a preovulatory primer pheromone, 17α20β-dihydroxy-4-pregnen-3-one (17,20β-P) and a postovulatory releaser pheromone, prostaglandin $F_{2\alpha}$ and its metabolites (PGFs; Stacey and Sorensen 1986; Stacey *et al.*, 1989). Since 17,20β-P induces final oocyte maturation (Goetz, 1983) and $PGF_{2\alpha}$ modulates follicular rupture and stimulates spawning behavior in female goldfish (see Stacey and Goetz, 1982), these two hormones also serve as pheromonal signals of the female's reproductive readiness and synchronize male-female reproductive physiology and behavior during spawning (see Sorensen and Stacey, 1989).

Based on the concept of pheromonal stimulation of pituitary GtH secretion in male goldfish, the present study in Chapter 5 would suggest that preovulatory 17,20β-P pheromones, by acting through the brain GnRH neuronal system, account for the serum GtH surges in males spawning with ovulating females. However, this notion is not supported by studies showing that exposure to water-borne 17,20β-P increases serum GtH levels, but did not alter serum GH levels or the GnRH concentrations in the telencephalon, hypothalamus and pituitary (Dulka *et al.*, 1989). Further studies comparing the endocrine responses of males to water-borne 17,20β-P with the responses to ovulating females under similar conditions are needed to clarify the involvement of pheromones in the activation of the brain GnRH system in male goldfish during spawning.

The observed differential changes of brain and pituitary GnRH levels in male goldfish in response to ovulating females (in Chapter 5) and to PG-treated females (in Chapter 3 and 4) clearly indicate that the regulation of brain GnRH activity under these two physiological conditions is different. Since ovulating females produce both 17,20β-P and PG pheromones while PG-treated females presumably release PG (but not 17,20β-P), the differential changes in brain and pituitary GnRH levels in males under these conditions supports the hypothesis that separate neuroendocrine mechanisms may be involved to

mediate the stimulatory effects of 17,20β-P and PG pheromones in pituitary GtH secretion in male goldfish (Sorensen *et al.*, 1987; 1988; Dulka, 1989). It is suggested that rapid GtH responses to 17,20β-P may involve the activation of a direct olfactory pathway to the preoptic area, causing a reduction of DA turnover in the pituitary (Dulka, 1989). On the other hand, stimulation of GtH release in response to PG pheromones may be mediated by another olfactory pathway to the preoptic area passing through the *area ventralis telencephali pars supercommissuralis* (Vs) and the *area telencephali pars ventralis* (Vv) which are known to be involved in the control of male sexual behavior (Kyle and Peter, 1982; Kyle *et al.*, 1982; Koyama *et al.*, 1984). Based on the findings in the present study that exposure to ovulating females (which presumably release both preovulatory 17,20β-P and postovulatory PG pheromones) or PG-treated females (which presumably release PG pheromones) alters the GnRH levels in various forebrain areas (in Chapters 3, 5), I further extend the hypothesis (Dulka, 1989) to propose that GnRH neurons in different brain areas (olfactory bulbs, ventral telencephalon including Vv and Vs, and preoptic region) are regulated, as an integrated unit, by these pheromonal inputs.

Considerable evidence suggests the participation of monoamines in the regulation of pituitary GtH secretion in goldfish (see Introduction, Section 1.2). The inhibitory effects of DA (Chang *et al*, 1984) and the stimulatory effects of norepinephrine (NE; Chang *et al*, 1984) and serotonin (5-HT; Somoza and Peter, submitted) on GtH release from pituitary fragments *in vitro* have been demonstrated in goldfish. Since goldfish pituitary fragments contain both GnRH nerve terminals and gonadotrophs, the sites of actions of the monoamines are not known from these studies. Other studies using cultured pituitary cells have demonstrated the direct actions of DA (Chang *et al.*, 1984, 1989; Chang *et al.*, submitted) and NE (Chang *et al.*, 1984) on pituitary GtH secretion in goldfish. However, little is known about the possible actions of monoamines in the regulation of GnRH secretion. Attempts to demonstrate central effects of monoamines in the regulation of pituitary GtH secretion by injection of DA, NE and 5-HT into the third ventricle of

goldfish revealed no significant effects by DA (Chang and Peter, 1983) and 5-HT (Somoza *et al*, 1988), and small stimulatory effects by one, but not a larger, dose of NE (Chang and Peter, 1984).

To examine the direct actions of monoamines on GnRH secretion, an *in vitro* static incubation system was developed for studying the release of GnRH from slices of the preoptic-anterior hypothalamus (P-AH) and fragments of the pituitary of goldfish (in Chapter 6). Using this *in vitro* approach, the present study has, for the first time, demonstrated an inhibitory and a stimulatory action of DA and 5-HT, respectively, on the release of GnRH from both the P-AH and pituitary in goldfish (Chapter 6). In addition, results in the present study also showed that NE has a stimulatory effect on GnRH release from the P-AH but not from the pituitary (Chapter 6 and 7). The presence of the monoaminergic and GnRH peptidergic structures in the preoptic region and pituitary provides the neuroanatomical substrate for a possible interaction at these two loci in teleosts (Kah and Chambolle, 1983; Yoshida *et al.*, 1983; Kah *et al.*, 1986; Hornby *et al.*, 1987; Kah *et al.*, 1987). However, definite morphological proof of such an interaction must await the demonstration of specific synaptic contacts in ultrastructural studies as described in mammals (see Silverman, 1988)

The involvement of specific dopaminergic and adrenergic receptors in modulation of GnRH release from the P-AH and pituitary in goldfish was further demonstrated in the present study (Chapter 7). The observation in goldfish that DA inhibits GnRH release through two pharmacologically distinct D_1- and D_2- dopaminergic receptors in the P-AH and pituitary respectively has two important implications. First, it supports and extends the previous hypothesis (in Chapter 4) that the central dopaminergic system inhibits brain GnRH activity by, at least, actions on the release mechanism. Secondly, the observation that a D_2 antagonist pimozide increases brain and pituitary GnRH levels (in Chapter 4) while it presumably blocks the inhibitory actions of D_2-receptors on GnRH secretion at the

level of pituitary supports the previous interpretation that pimozide may stimulate the biosynthesis and/or decrease in degradation, more than it stimulates the rate of transport or release, of GnRH peptides in the goldfish brain. Although more studies are required to elucidate the physiological significance of the dopaminergic (D_1 and D_2), adrenergic (α_1) and serotonergic modulation of GnRH secretion, the present study has provided original information clearly demonstrating the involvement of various monoamines in the regulation of GnRH release from the P-AH and pituitary in a teleost.

Previous study has demonstrated at least two immunoreactive GnRH forms in extracts of goldfish brain; one form of GnRH was characterized as sGnRH based on HPLC elution profiles (Sherwood and Harvey, 1986). The important question as to whether both of these GnRH forms are present in the pituitary and different parts of the brain remains unanswered. By using a combined HPLC and immunological analysis with a library of antisera crossreacting differently with the five known vertebrate GnRH peptides, the molecular heterogeneity of the immunoreactive GnRH in each discrete brain area and the pituitary of both male and female goldfish was examined in the present study (Chapter 8). Using this approach, the second form of GnRH in goldfish brain and pituitary was characterized to be cGnRH-II (Chapter 8). The ultimate proof of the identity of the second form of GnRH in goldfish brain as cGnRH-II still relies on the elucidation of the amino acid sequence. However, this combined HPLC and immunological approach has been used by other workers (King and Millar, 1982) to successfully predict the primary structure of one of the GnRH forms ([Gln^8]-GnRH, chicken GnRH-I or cGnRH-I) in the chicken brain before confirmation by amino acid sequence analysis.

In addition, the present study demonstrates, for the first time, that the two GnRH forms are differentially distributed in the goldfish brain (Chapter 8); sGnRH is preferentially more concentrated in the pituitary and forebrain areas compared to the caudal brain areas whereas cGnRH-II is more evenly distributed throughout the brain and

pituitary. This concept of differential distribution of multiple GnRH forms in brains of vertebrates is supported by results from RIA and immunocytochemical studies in chicken (Hasegawa *et al.*, 1986) and Japanese quail (Mikami *et al.*, 1988). Studies in male chicken showed that caudal brain areas have a much higher content of cGnRH-II, whereas the hypothalamus and preoptic region contained mainly cGnRH-I (Hasegawa *et al.*, 1986). Immunocytochemical studies in Japanese quail further showed that immunoreactive cGnRH-I (ir-cGnRH-I) cell bodies were found in the preoptic region, and ir-cGnRH-I fibers in the ME; on the other hand, immunoreactive cGnRH-II (ir-cGnRH-II) fibers are absent from the ME, and ir-cGnRH-II cell bodies are detectable mainly in the midbrain. In contrast to the situations in these two avian species (chicken and Japanese quail), both GnRH forms are present in the goldfish pituitary suggesting that both forms may have a neuroendocrine role in the pituitary of goldfish in addition to the possible neurotransmitter functions in the brain.

In conclusion, the results of the present investigation make it possible to construct models describing the environmental (Fig. 9.1) and monoaminergic (Fig. 9.2) inputs to the GnRH neuronal system in the goldfish. The GnRH neurons in various parts of the forebrain areas form a functional unit to integ and transduce environmental (temperature, photoperiod, pheromones, spawning partner, spawning substrate) and physiological (eg. gonadal steroid feedback signals) inputs. The preoptico-hypophyseal GnRH tract represents the neuroendocrine pathway that mediates the GnRH regulation of pituitary GtH and possibly also GH secretion. The activity of this integrated GnRH unit is altered during spawning in male (Chapter 5) and female (Chapters 2 and 5) goldfish, and during spawning behavior with PG-treated females in male goldfish (Chapters 3 and 4). The central dopaminergic system has an inhibitory action on brain and pituitary GnRH levels by presumably inhibiting the biosynthesis and/or stimulating degradation of the peptide (Chapter 4). In addition, DA inhibits GnRH release at the level of preoptic-anterior hypothalamic region and pituitary through D_1- and D_2- dopaminergic receptors,

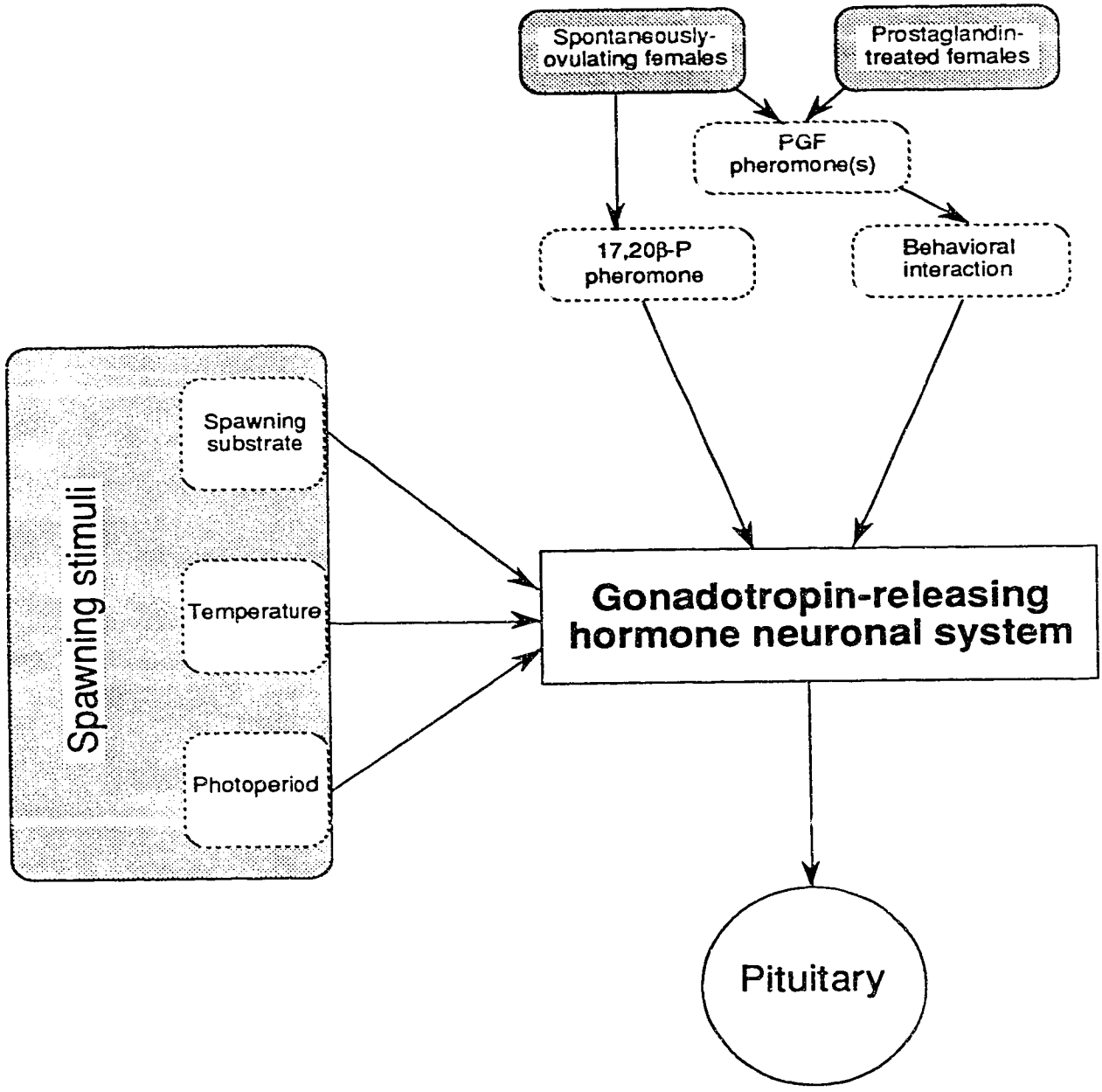

Fig. 9.1. Environmental inputs to the gonadotropin-releasing hormone neuronal system in the goldfish. See text for abbreviations and detailed explanations.

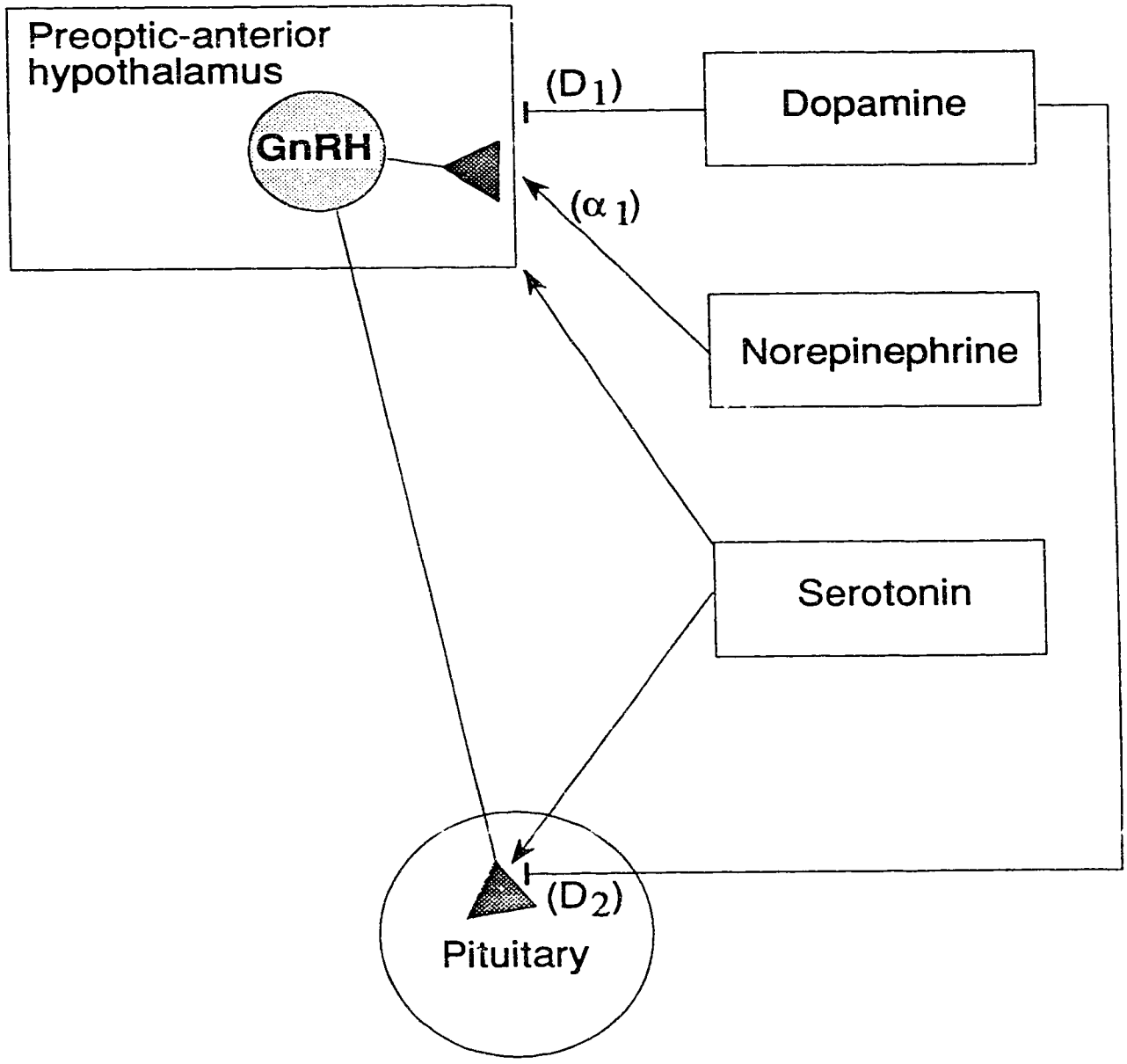

Fig. 9.2. Monoaminergic inputs to the gonadotropin-releasing hormone (GnRH) neuronal system in the goldfish. Stimulatory pathways are indicated by lines ending with an arrow; inhibitory pathways are indicated by lines ending with a solid bar. See text for detailed explanations.

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