Reviews of Reproduction (1997) **2**, 55–68

Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, *Carassius auratus*

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The goldfish, a member of the carp family, is a widely used model for reproductive neuroendocrine studies of economically important fish. The two gonadotrophin (GTH) molecules released from the fish anterior pituitary, GTH-I and GTH-II, are structurally similar to tetrapod FSH and LH, respectively. Gonadotrophin II is the best studied, and in goldfish stimulates gonadal growth and steroidogenesis, ovulation and sperm release. Growth hormone also has gonadotrophic actions in fish which enhance gonadal steroidogenesis. The principal stimulatory and inhibitory systems regulating GTH-II release are the gonadotrophin-releasing hormone (GnRH) and dopamine neurones in the preoptic–hypothalamic region. In goldfish there are two native GnRH forms, salmon GnRH and chicken GnRH-I; both stimulate GTH-II release but use different signal transduction pathways. In contrast to mammals, teleost fish do not have a median eminence and the GTH-II cells are thus directly innervated by neurones producing GnRH, dopamine and other stimulatory neurohormones. For most of these factors, the ability to stimulate GTH-II release varies seasonally. The amino acid neurotransmitter, γ-aminobutyric acid, has the most prominent stimulatory actions which enhance GnRH release and inhibit dopamine turnover in the hypothalamo–pituitary complex. Neuropeptide Y stimulates GTH-II release by a combined direct action on the gonadotroph and also by enhancing GnRH release. Positive and negative sex steroid feedback mechanisms act concurrently to regulate GTH-II release in adults of both sexes. The principal site of positive feedback is the GTH-II cell where testosterone and oestradiol potentiate GnRH-stimulated GTH-II release. Negative feedback by sex steroids involves activation of inhibitory dopamine neurones, thus maintaining tight control over circulating GTH-II concentrations.

Of all the vertebrate classes, teleosts, or bony fishes, are the most numerous, both in individuals and in species (approximately 20 000 teleosts versus 4 000 mammals). They occupy a variety of aquatic environments and show an incredible diversity in reproductive strategies. For many, this requires seasonal reproduction to ensure that breeding coincides with optimal water temperatures and food supplies for developing offspring. The annual cycle in gonadal growth and function is controlled by circulating concentrations of the gonadotrophic (GTH) hormones released from the pituitary. The morphology of the teleost hypothalamo–pituitary axis has been elegantly reviewed on several occasions (Peter *et al.*, 1990; Gorbman, 1995) but several interesting features should be briefly mentioned. A major difference between teleosts and mammals is that teleosts do not have a functional hypothalamo–hypophyseal portal blood system. The teleost adenohypophysis has three distinct lobes: the rostral pars distalis; the proximal pars distalis; and the neurointermediate lobe, which is composed of the pars intermedia with interdigitation and innervation by the homologue of the posterior pituitary or caudal neurohypophysis. The anterior neurohypophysis, homologous to the tetrapod median eminence, innervates the rostral and proximal regions of the pars distalis. Distribution of the various endocrine cells in the teleost pituitary is highly regionalized, in contrast to a more dispersed organization in mammals. The proximal pars distalis contains almost exclusively gonadotrophs and somatotrophs. The rostral pars distalis contains primarily prolactin cells but also thyrotrophs, and corticotrophs, which are often localized at the border between the rostral and proximal pars distalis. There are two major GTH molecules secreted from different cells in the proximal pars distalis in fish, GTH-I and GTH-II, which are structurally similar to tetrapod FSH and LH, respectively (Quérat, 1994; Schulz *et al.*, 1995). GTH-I has only recently been discovered and stimulates gonadal steroidogenesis (Quérat, 1994; VanDerKraak and Wade, 1994); however, there is no information on its neuroendocrine control. In contrast, there is extensive data for GTH-II which stimulates gonadal steroidogenesis, gametogenesis and ovulation (or sperm release), and is thus an important regulator of fertility (Peter *et al.*, 1991; Kah *et al.*, 1993). Analogous to mammalian FSH and LH receptors, a two-receptor model for GTH-I and GTH-II action on fish gonads has been proposed (Yan *et al.*, 1992). VanDerKraak and Wade (1994) have presented the comparative aspects of gonadotrophic hormone action in the vertebrates. In the goldfish model, seasonal variations in gonadal size are highly correlated with basal serum GTH-II concentrations (Fig. 1). Serum growth hormone (GH) concentrations are also high when gonadal size is increasing in the winter, when somatic growth is actually very slow. This suggests that GH may also be important in controlling seasonal reproduction in goldfish.

1359-6004/97 $10.00

Teleost fish are an excellent model for the study of positive neuroendocrine control of reproduction. The proximal pars distalis of the teleost pituitary is heavily innervated by neurones synthesizing a number of neuropeptides and neurotransmitters (Peter *et al*., 1990; Kah *et al*., 1993) that appear to stimulate GTH-II release (Fig. 2). The teleost model of direct innervation of the pars distalis also allows precise determination of the entire hypophysiotrophic system using neuronal tract tracing techniques (Anglade *et al*., 1993), which is much more difficult in animals with a median eminence. The primary goal of this review is to provide an integrated, up-to-date view of the major neuroendocrine systems controlling GTH-II release in relation to seasonal gonadal development in a teleost fish. The goldfish *(Carassius auratus)* is considered in detail, while notable differences and similarities with other species will be highlighted. Several other excellent reviews are available and the reader is directed to Kah *et al.* (1993), Yaron (1995) and Schulz *et al.* (1995) for alternative points of view.

Evidence that gonadotrophin releasing hormone and dopamine are physiological regulators of gonadotrophin II release

The decapeptide, GnRH, stimulates GTH-II release in fish in a similar way to GnRH-stimulated LH release in other vertebrates (Chang and Jobin, 1994). Nine vertebrate GnRHs have been isolated and are named according to the species in which the amino acid sequence was first determined. In most non-mammalian species, multiple GnRH forms are found in brain. In fish, GnRH occurs in at least two genomic isoforms, and the molecular evolution and biological implications of multiple GnRH forms in one species have been reviewed (Schulz *et al*., 1993a; Sherwood *et al*., 1993, 1994; Chang and Jobin, 1994). The observation that is relevant here is that the two co-exisiting GnRHs in goldfish are salmon GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2) and chicken GnRH-II (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2), both found in pituitary nerve terminals, suggesting that more than one native GnRH peptide stimulates GTH-II release *in vivo* (Chang and Jobin, 1994). Indeed, detailed structure–activity studies of native GnRHs and multiple GnRH agonists and antagonists confirm this (Peter *et al*., 1991; Habibi and Pati, 1993; Murthy *et al*., 1993). The presence of two functional GnRHs in one species raises interesting questions about GnRH receptor–signal transduction pathways. Current evidence in goldfish indicates the existence of one high affinity (K_a~10^{10} M^{-1}) pituitary GnRH receptor mediating biological activity and one low affinity (K_a~10^7 M^{-1}) GnRH receptor with no known function (Habibi *et al*., 1989; Habibi and Pati, 1993). Chang and colleagues discovered that there are major differences in the mechanisms of sGnRH and cGnRH-II action to stimulate GTH-II release (Chang *et al*., 1993; Chang and Jobin, 1994). Briefly, the two GnRHs similarly enhance inositol phosphate turnover, diacylglycerol production and protein kinase C activity (Fig. 3). However, they differ in their calcium and arachidonic acid requirements for signal transduction. cGnRH-II is much more dependent on entry of extracellular calcium through a voltage sensitive calcium channel than is sGnRH. Moreover, mobilization and metabolism of arachidonic acid through a lipoxygenase pathway is required for sGnRH but not cGnRH-II

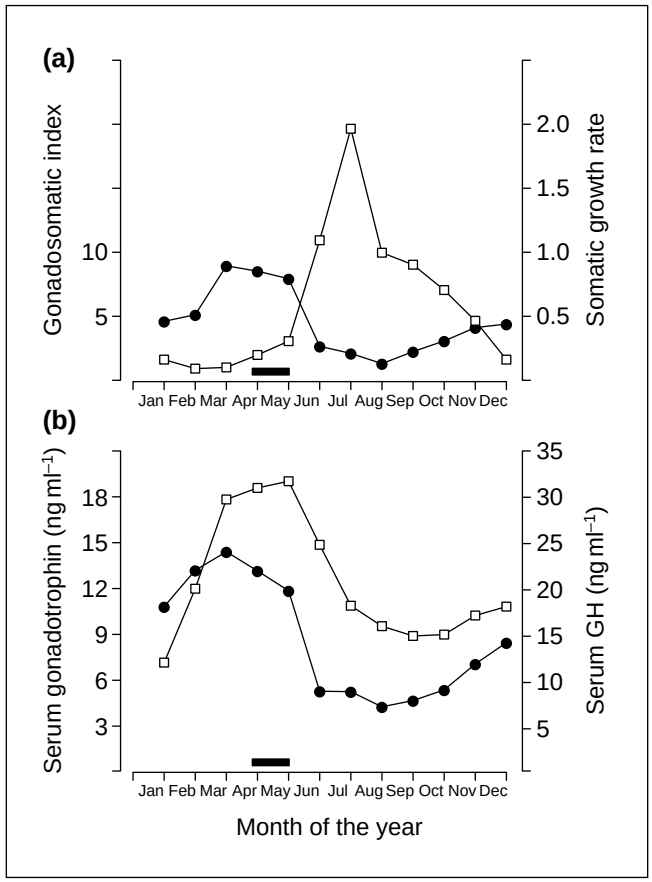


Fig. 1. Seasonal variations in reproduction and growth in female goldfish. (a) Seasonal changes in gonadosomatic index (●; gonad mass/body mass × 100) and somatic growth rate (□; percentage increase in somatic weight per day). (b) Seasonal changes in serum gonadotrophin II (●) and growth hormone (□) concentrations. Data are adapted from Marchant and Peter, 1986 and Trudeau *et al*., 1991b. The approximate spawning season in temperate climates is shown by ▬.

stimulated GTH-II release (Chang and Jobin, 1994). Thus, in goldfish at least, two native GnRHs appear to compete for a similar set of high-affinity receptors, but how they activate different signal transduction pathways is unknown. It is also known that sGnRH and cGnRH-II have different distributions in the brain. High performance liquid chromatography and radioimmunoassay studies on brain extracts initially indicated that sGnRH predominates in the goldfish forebrain, whereas cGnRH-II predominates in more caudal brain regions (Yu *et al*., 1988). Subsequent neuroanatomical studies (Kim *et al*., 1995) indicate that sGnRH cell bodies are found in the ventral telencephalon, preoptic area and hypothalamus. Although less numerous than sGnRH cells, cell bodies immunoreactive for cGnRH-II have a similar distribution. In addition, a small cluster of neurones in the nucleus of the medial longitudinal fasciculus (midbrain tegmentum) produce cGnRH-II. Fibres for both sGnRH and cGnRH-II are found in the preoptic–hypophyseal tract and also throughout the entire brain. This extensive GnRH innervation suggests that in goldfish, both GnRH forms not only regulate GTH-II release but also have other important

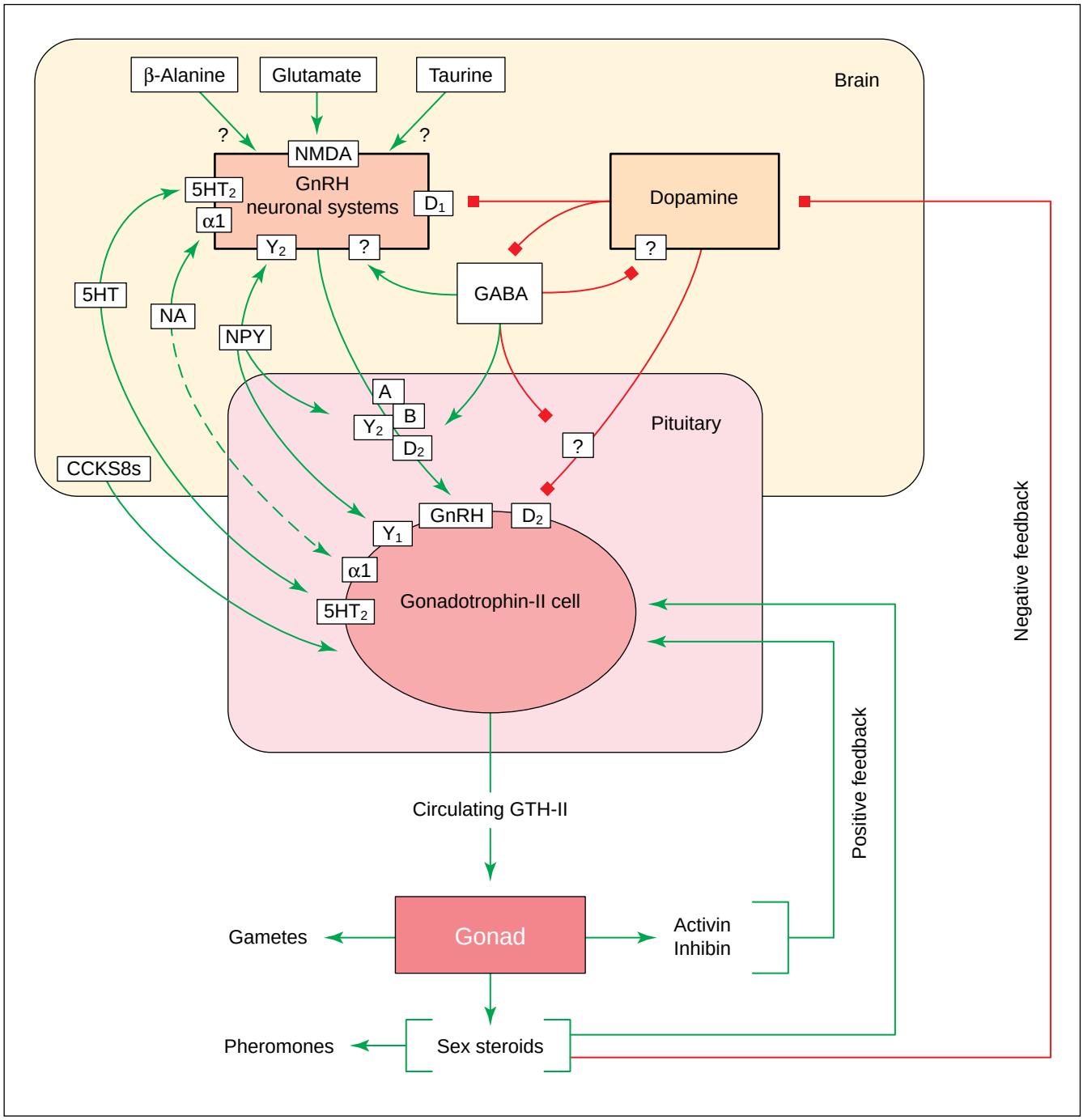


Fig. 2. Multifactorial control of gonadotrophin II (GTH-II) release in goldfish. Multiple stimulatory factors have been found to enhance pituitary GTH-II release. Central to the control of GTH-II release are salmon gonadotrophin-releasing hormone (sGnRH) and chicken GnRH-II (cGnRH-II). Represented here is a combined 'GnRH neuronal system' because most studies have not yet addressed differential release of the two GnRH forms. Dopamine is the only inhibitory neurohormone identified to date. GTH-II from the gonadotrophs in the pars distalis of the pituitary is released into the circulation to regulate gonadal function. Positive and negative feedback mechanisms act concurrently to regulate finely GTH-II release. Sex steroids enhance pituitary responsiveness to sGnRH and cGnRH-II and neuropeptide Y (NPY), and also increase the inhibitory effects of dopamine on GTH-II release. α1, alpha-1 noradrenergic receptor; A, type A γ-aminobutyric acid receptor; B, type B γ-aminobutyric acid receptor; CCK8s, sulfated cholecystokinin-8; D_1, type-1 dopamine receptor; D_2, type-2 dopamine receptor; GABA, γ-aminobutyric acid; 5HT, 5-hydroxytryptamine; $5HT_2$, type-2 5-hydroxytryptamine receptor; NA, noradrenaline; Y_1, type-1 neuropeptide Y receptor; Y_2, type-2 neuropeptide Y receptor. Factors tested and found to have no effects on GTH-II release are catecholoestrogens, melatonin, cysteic acid, glutamine, isetheonic acid, lysine, non-sulfated CCK-8, growth hormone-releasing hormone, thyrotropin-releasing hormone and somatostatin. ➤: stimulation; ⊣: inhibition. Note: noradrenaline can directly stimulate (⇢) GTH-II release from the gonadotroph but has not been detected in the pituitary. Peripherally released noradrenaline may therefore be the endogenous stimulus.

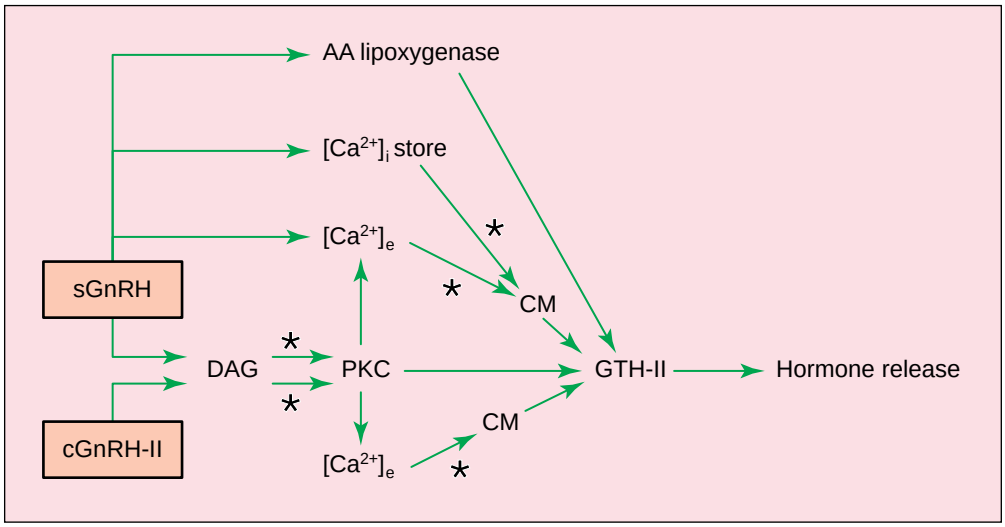


Fig. 3. Major intracellular signal transduction pathways known to mediate salmon GnRH (sGnRH) and chicken GnRH-II-stimulated gonadotrophin II (cGTH-II) release from dispersed goldfish pituitary cells *in vitro*. * Indicates possible sites for dopaminergic inhibition of stimulated GTH-II release. The ➤ indicates stimulation. AA, arachidonic acid; $[Ca^{2+}]_e$, extracellular calcium entry through a voltage-sensitive calcium channel; $[Ca^{2+}]_i$, intracellular calcium stores; CM, calmodulin; DAG, diacylglycerol; PKC, protein kinase C. Data are modified from Chang *et al.*, 1993 and Chang and Jobin, 1994.

neuromodulatory functions. In addition to controlling GTH-II release through different signal transduction pathways, the differential distribution of sGnRH versus cGnRH-II immunoreactive cell bodies raises the possibility that the two cell populations could be regulated by different neurotransmitters or respond to different physiological signals. These suggestions await rigorous testing.

In fish, dopamine is the only inhibitory neurohormone identified to date. The origin of inhibitory dopaminergic neurones projecting to the pituitary is the preoptic area, specifically the ventral and lateral walls of the preoptic recess (Kah *et al.*, 1993). Extensive pharmacological studies indicate that dopamine is released from nerve terminals in the goldfish pituitary where it activates dopamine D_2 receptors on the gonadotroph to restrain GTH-II secretion directly, and also by decreasing GnRH receptor binding to the pituitary (Peter *et al.*, 1986; Habibi *et al.*, 1989; Sloley *et al.*, 1991, 1992a). Dopamine can also interfere with intracellular GnRH signal transduction pathways to inhibit GTH-II release (Fig. 3). Principally, dopamine reduces extracellular calcium influx and inhibits PKC-dependent mechanisms, consistent with the known transduction mechanisms of D_2-like receptors in mammals (Chang *et al.*, 1993). Dopamine has additional inhibitory effects on GnRH release at the pituitary nerve terminal through a D_2 receptor, and within the preoptic region through a D_1-like receptor (Peter *et al.*, 1990; Yu and Peter, 1990, 1992). Thus, through multiple pathways and mechanisms, dopamine is central to the inhibitory control of GTH-II release.

Several observations on natural changes in GTH-II release emphasize the physiological roles of the GnRH and dopamine systems. The steroidal pheromone 17α,20β-dihydroxy-4-pregnen-3-one, which is released by female goldfish into water (Stacey *et al.*, 1994), is a potent olfactory cue that stimulates GTH-II release in males by activation of GnRH neurones (Yu *et al.*, 1991a; Murthy *et al.*, 1994) and inhibition of pituitary dopamine turnover (Dulka *et al.*, 1992). Moreover, in the female ciclid fish, *Haplochromis burtoni*, GnRH cell size increases during natural reproductive development (White and Fernald, 1993). Other important neuroendocrine factors found to stimulate GTH-II release are highlighted below.

Amines

In contrast to the prominent inhibitory effects of dopamine, noradrenaline has only a slight stimulatory effect on GTH-II release via an α-1 receptor mechanism; sexually regressed goldfish are more responsive than mature animals (Chang *et al.*, 1991). Noradrenaline innervation of the pituitary has not been detected (Dulka *et al.*, 1992; Trudeau *et al.*, 1993a); thus peripherally released noradrenaline probably acts on GnRH nerve terminals and directly on the gonadotrophs to stimulate GTH-II release. Centrally released noradrenaline may activate the GnRH neurone (Peter *et al.*, 1990, 1991; Yu *et al.*, 1991b). 5-Hydroxytryptamine (5HT) stimulates GTH-II release via a $5HT_2$-like receptor in goldfish (Somoza and Peter, 1991) and Atlantic croaker (Khan and Thomas, 1994) by activating the GnRH neurone at either the cell body or terminal (Yu *et al.*, 1991b). Pineal melatonin is synthesized from 5HT but melatonin has no direct effects on pituitary GTH-II release (Somoza and Peter, 1991). A role for melatonin in seasonal photoperiodic control of reproductive cycles has long been postulated in fish but strong evidence is still lacking. However, it remains possible that melatonin is important because its secretion is regulated by photoperiod, and melatonin receptors are found in neuroendocrine territories of the teleost brain (Martinoli *et al.*, 1991; Kah *et al.*, 1993). Goldfish have a circadian ovulatory cycle in the spring breeding season which is determined by a photoperiodic cue (Aida, 1988) but it is not known whether melatonin is involved.

Amino acids

In goldfish, the glutamate agonist *N*-methyl-D,L-aspartic acid (NMDA) stimulates GTH-II release. The GTH-II response to NMDA is generally small and is not affected by sex steroids (Trudeau *et al.*, 1993b). These observations in goldfish contrast with the majority of data in mammals in which glutamate is a prominent stimulator of GnRH and LH release, and sex steroids potentiate NMDA action (Brann and Mahesh, 1994). In trout, NMDA stimulates GTH-II secretion by enhancing GnRH release (Flett *et al.*, 1994), and a similar mechanism is likely to occur in goldfish. Whether glutamate can stimulate GTH-II release through activation of non-NMDA receptors has not been determined in any fish. The sulfur-containing amino acid, taurine, is found in substantial amounts in goldfish brain and pituitary, and both intraperitoneal and brain injections of taurine stimulate GTH-II release in goldfish (Sloley *et al.*, 1992b; Trudeau *et al.*, 1993b). High concentrations of injected taurine are needed to stimulate GTH-II release, which reflects its abundance in neuroendocrine tissues. Hypotaurine, the immediate precursor for taurine biosynthesis, but not the metabolite isetheonic acid, stimulates GTH-II release in goldfish (Sloley *et al.*, 1992b). Both taurine- and hypotaurine-stimulated GTH-II release is potentiated by testosterone (Trudeau *et al.*, 1993b), further implicating taurine in the control of fish reproduction. Taurine decreases LH secretion in female rats via inhibition of hypothalamic GnRH release (Arias, 1995). In goldfish, taurine could stimulate the GTH-II release directly, or indirectly, through activation of sGnRH or cGnRH-II or by inhibition of dopaminergic neurones. β-Alanine has also been shown to stimulate GTH-II release *in vivo* (Sloley *et al.*, 1992b) but its presence in goldfish brain or pituitary, or a physiological role for this amino acid has not been determined.

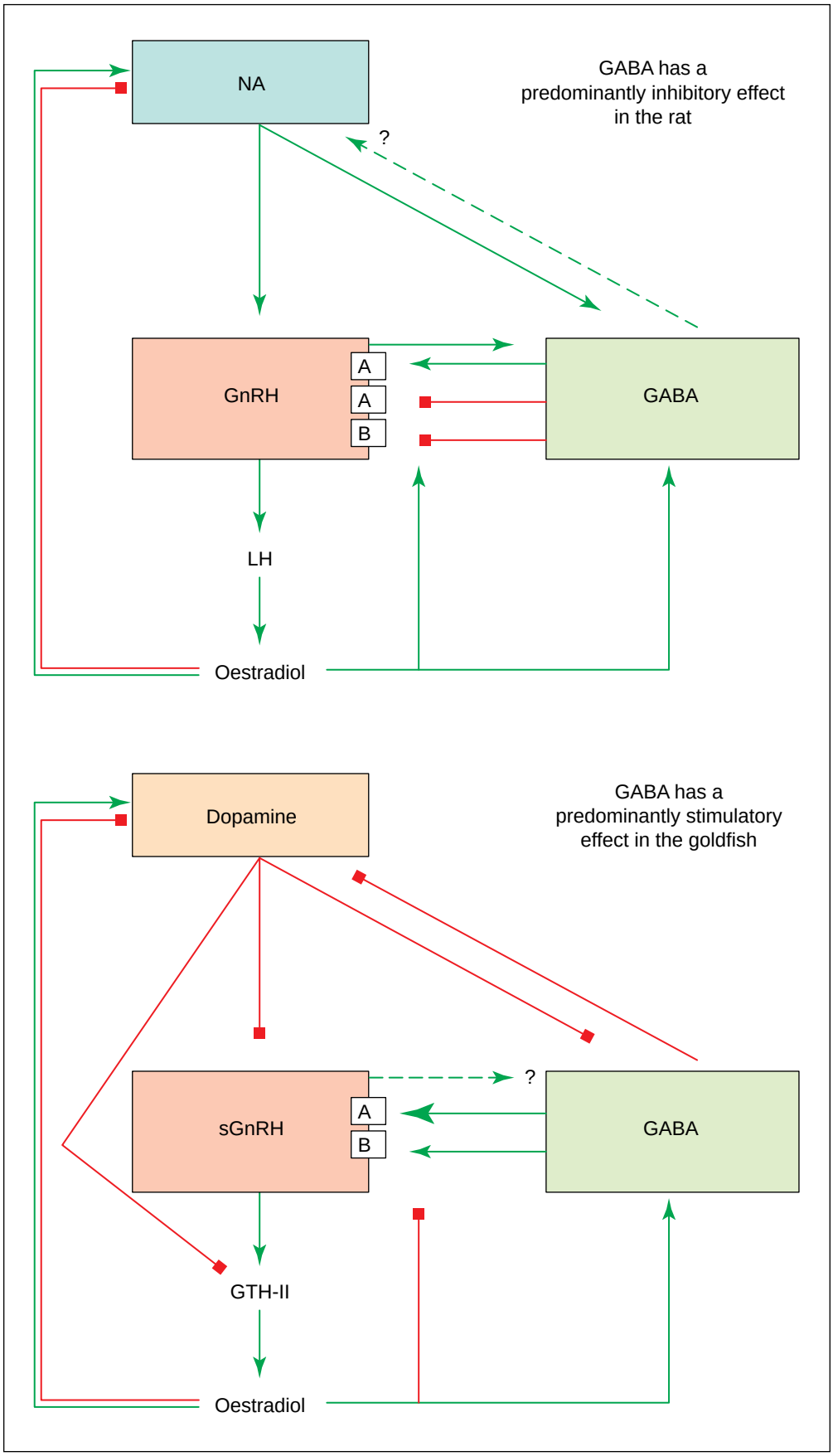
Gamma-aminobutyric acid (GABA) immunoreactive perikarya are found in the major neuroendocrine territories of the goldfish telencephalon and hypothalamus, and GABA neurones directly innervate the anterior pituitary (Martinoli *et al.*, 1990; Kah *et al.*, 1993). The origin of GABAergic innervation of the pituitary has not been established, but could be the preoptic area or the nucleus lateralis tuberis (the teleost homologue of the mammalian arcuate nucleus), which are both hypophysiotrophic centres (Anglade *et al.*, 1993) containing GABA immunoreactivity (Martinoli *et al.*, 1990). Brain third ventricle and intraperitoneal injections of GABA stimulate GTH-II release in goldfish (Kah *et al.*, 1992; Trudeau *et al.*, 1993b, c), which contrasts with most data in mammals in which GABA has a predominant inhibitory effect on LH release. The stimulatory effects of GABA on GTH-II release in goldfish results from both increased GnRH release and decreased dopaminergic activity (Trudeau *et al.*, 1993c), but not by direct action on basal or GnRH-stimulated GTH-II release from the gonadotroph (Kah *et al.*, 1992). Current evidence indicates that the principal mechanism of GABA action is through the GnRH system since inhibition of dopamine synthesis and pretreatment with the dopamine antagonist, domperidone, does not block GABA action (Trudeau *et al.*, 1993c). GABA is synthesized by decarboxylation of glutamate and is metabolized to succinic acid by GABA transaminase. Inhibition of GABA transaminase by γ-vinyl-GABA (GVG) raises endogenous brain and pituitary GABA concentrations and stimulates GTH-II release in goldfish (Sloley *et al.*, 1992b). Anti-dopaminergic treatments potentiate GVG-stimulated GTH-II release (Trudeau, *et al.*, 1993c). Associated with GVG-stimulated hypersecretion of GTH-II is depletion of hypothalamic stores of sGnRH but not cGnRH-II, indicating that GABA preferentially regulates sGnRH neurones over those synthesizing cGnRH-II (Sloley *et al.*, 1994). Muscimol (a $GABA_A$ receptor agonist) stimulates GTH-II release, and bicuculline (a $GABA_A$ receptor antagonist), but not saclofen (a $GABA_B$ receptor antagonist), blocks GABA-stimulated GTH-II release *in vivo* in goldfish. These results indicate the involvement of a $GABA_A$-like receptor (Trudeau *et al.*, 1993c). Moderate doses of baclophen (a $GABA_B$ receptor agonist) can also stimulate GTH-II, indicating that a $GABA_B$ receptor-mediated mechanism may also be important *in vivo* (Trudeau *et al.*, 1993c). Preliminary data indicate that the stimulatory effects of GABA are not limited to goldfish: GABA stimulates pituitary GTH-II accumulation in European eels (I. Mayer and S. Dufour, unpublished) and GTH-II release in African catfish (R. Schulz and C. Johnson, unpublished). Together these data indicate the physiological importance of endogenous GABA in stimulating GTH-II secretion in fish.

GABA regulated GTH-II release in goldfish is different from GABA-regulated LH release in rats (Fig. 4). In rats, drugs that affect the $GABA_A$ receptor both stimulate and inhibit the release of LH *in vivo* and GnRH *in vitro* (see Favit *et al.*, 1993 and Brown *et al.*, 1994, for examples), although the widely accepted view is that principally GABA inhibits LH release. Using the GnRH secreting GT-1 neuronal cell line, Favit *et al.* (1993) showed that $GABA_A$ receptor activation stimulates basal GnRH release, whereas $GABA_B$ receptor activation inhibits depolarization-induced GnRH release. GABA, through activation of a biccuculine-insensitive, nonclassical $GABA_A$ receptor, also stimulates LH release directly from the rat gonadotroph *in vitro* (Virmani *et al.*, 1990), in contrast to the lack of effect of GABA on GTH-II release from dispersed goldfish gonadotrophs (Kah *et al.*, 1992). However, Kah *et al.* (1993) presented preliminary evidence that GABA and muscimol stimulate intracellular Ca^{2+} mobilization in isolated goldfish gonadotrophs, raising the possibility that GABA may have an additional direct effect in some circumstances.

The significance of the differences in GABA action to enhance GTH-II release in fish versus the predominantly inhibitory effects of GABA on LH release observed in mammals is unknown. One hypothesis is that the stimulatory effects of GABA on gonadotrophin secretion in both fish and mammals represents the older evolutionary mechanism. The inhibitory effects of GABA observed in mammals but not fish could represent a more recently evolved control mechanism. Clearly, more comparative studies and full characterization of GABA receptors in fish are needed to establish the broader context in which GABA can stimulate reproductive function.

Neuropeptides and growth factors

Cholecystokinin (CCK) stimulates GTH-II release from goldfish pituitary fragments *in vitro*; CCK must be in its sulfated form, CCK8s, to be active (Himick *et al.*, 1993). Sexually mature animals are more responsive to CCK8s than are sexually regressed fish. Naloxone, an opiate receptor antagonist, has both stimulatory and inhibitory effects on GTH-II release in goldfish


NA
GABA has a predominantly inhibitory effect in the rat
?
GnRH
A
A
B
GABA
LH
Oestradiol
Dopamine
GABA has a predominantly stimulatory effect in the goldfish
?
sGnRH
A
B
GABA
GTH-II
Oestradiol

in vivo (Rosenblum and Peter, 1989), suggesting that the endogenous opiates have a modulatory role in controlling GTH-II secretion. Neuropeptide Y (NPY) stimulates GTH-II release in goldfish through a Y_2-like receptor mechanism to enhance GnRH release, and also by direct stimulation of Y_1-like receptors on the gonadotroph (Peng *et al.*, 1993a, b).

Recent evidence indicates that both inhibin and activin stimulate GTH-II release from goldfish pituitaries *in vitro* (Ge *et al.*, 1992). This contrasts with mammalian data showing that inhibin and activin, respectively, inhibit and stimulate FSH release, while rarely affecting LH release in most species (Mather *et al.*, 1992). Cloning and sequencing of goldfish activin subunit genes from the ovary confirm that gonadal tissue is one site for the synthesis of inhibin/activin-like peptides in fish, as in mammals (Ge *et al.*, 1993). There are similarities between goldfish and mammalian molecules: goldfish βA subunit has 78% protein sequence identity with human βA, and βB subunits have more than 94% identity (Ge *et al.*, 1993). In addition to gonadal activin, somatotrophs in the pituitary produce activin and may exert stimulatory paracrine control over nearby GTH-II cells in the pars distalis of the pituitary (Ge and Peter, 1994).

Mechanisms of sex steroid feedback control of pituitary gonadotrophin II release

Gonadectomy and sex steroid replacement experiments have demonstrated classic negative feedback in several fish species (for review see Kobayashi and Stacey, 1990; Trudeau *et al.*, 1991b, c, 1993d; Kah *et al.*, 1993). It was previously thought that positive steroidal feedback was found only in prepubertal fish, particularly members of the salmon family (Xiong *et al.*, 1994) or in European eels (Fontaine and Dufour, 1991), in which sex steroids stimulate the synthesis and accumulation but not the release of GTH-II. Recent evidence in adult gonad-intact goldfish, however, indicates that there is a potent positive sex steroid feedback loop to control GTH-II release. The GTH-II-releasing activity of many neuropeptides and neurotransmitters is enhanced by sex steroids that contribute to gonadal feedback and seasonal cyclicity of the various neuroendocrine systems. To illustrate this, four systems will be considered in detail: GnRH, neuropeptide Y, dopamine and GABA.

Gonadotrophin releasing hormone

Pituitary responsiveness to native GnRH molecules and several GnRH analogues is positively correlated with gonadal size; maximal GTH-II release occurs in sexually mature animals and minimum GTH-II release occurs in immature animals (Habibi *et al.*, 1989; Trudeau *et al.*, 1991b). The seasonal increase in GnRH responsiveness in goldfish is associated with increased pituitary GnRH receptor binding (Habibi *et al.*, 1989) and pituitary GTH-II content in sexually mature animals (Trudeau *et al.*, 1991b). There are also seasonal variations in GnRH-stimulated expression of mRNA encoding glycoprotein α– and GTH-IIβ–subunits *in vivo* (Khakoo *et al.*, 1994). Salmon GnRH stimulates mRNA encoding both α– and GTH-IIβ–subunits, and cGnRH-II is without effect in sexually regressed goldfish. In contrast, both sGnRH and cGnRH-II stimulate expression of mRNA encoding α– and GTH-IIβ in mature animals. In male and female gonad-intact goldfish, testosterone, through aromatization to oestradiol, enhances pituitary GnRH responsiveness both *in vivo* and *in vitro* (Trudeau *et al.*, 1991b, c, 1993d). The stimulatory effect of sex steroids is part of a positive feedback loop since injection of hCG in sexually mature male goldfish increases steroid production and enhances GnRH responsiveness *in vivo* (Trudeau *et al.*, 1991b). Experiments *in vitro* have demonstrated that testosterone acts directly at the pituitary to enhance GnRH responsiveness (Lo *et al.*, 1995). The short term effects of testosterone in potentiating GnRH-stimulated GTH-II release is protein synthesis-dependent, but does not involve a change in pituitary GTH-II content or GnRH-receptor capacity (Trudeau *et al.*, 1993d). In goldfish the androgens, 5α-dihydrotestosterone and 11-ketotestosterone (an abundant androgen in fish; see Borg, 1994), have no effects on basal or GnRH-stimulated GTH-II release *in vivo* (Trudeau *et al.*, 1991b, 1993d). In sexually regressed goldfish, progesterone has no effect alone but potentiates the positive effect of oestradiol on GnRH-stimulated GTH-II release (Trudeau *et al.*, 1991c). The positive effects of testosterone on GTH-II secretion have also been observed in another member of the Cyprinidae, the common carp, and in an unrelated species, the Chinese loach, suggesting that positive feedback control of GTH-II release is a common feature in adult teleost fish (Trudeau *et al.*, 1991a). Inhibitory effects of sex steroids have been reported in some species of fish (Habibi *et al.*, 1989; Schulz *et al.*, 1993b) and differences in GTH-II responses may

Fig. 4. Comparison of the mechanisms of γ-aminobutyric acid (GABA) action in rats and goldfish. Luteinizing hormone (LH) release is controlled by the interaction of a catecholamine, noradrenaline (NA) and GABA with GnRH neurones. Physiological and computer modelling studies in rats (see Favit *et al.*, 1993 and Brown *et al.*, 1994 for examples) have established the probable interactions between the neurotransmitter systems. In a comparable manner, gonadotrophin II (GTH-II) release in goldfish is controlled by the interaction of a catecholamine, dopamine (DA) and GABA with salmon GnRH (sGnRH) neurones (Trudeau *et al.*, 1993a, c). However, the nature of the dopaminergic neurone is solely inhibitory on GTH-II release, which must be contrasted to noradrenaline in rats, which has both stimulatory and inhibitory effects on LH release. In rats GABA can activate both $GABA_A$ (A) and $GABA_B$ (B) receptors on the GnRH neurone. This has a dominant inhibitory effect, although $GABA_A$ receptor activation can stimulate GnRH release in some circumstances. In contrast, activation of the $GABA_A$ receptor in goldfish only stimulates GTH-II release, and activation of the $GABA_B$ receptor has a less pronounced, but nevertheless stimulatory, effect on GTH-II release. The nature of the GABA receptor subtypes mediating GABA–noradrenaline interactions in rats or GABA–dopamine interactions in goldfish are not known. Direct evidence of GABA receptor localization on the sGnRH neurones in goldfish brain is still lacking. Oestradiol and other steroids modulate GABA action in both species. In rats, oestradiol can enhance GABA release and GABA-inhibited LH release *in vivo*. Oestradiol modulates noradrenergic inputs to rat GABA neurones. In goldfish, oestradiol increases GABA synthesis but inhibits GABA-stimulated GTH-II release. Oestradiol also modulates the activity of dopaminergic neurones in goldfish. ➤ : stimulation and ⊣ : inhibition.

be due to differences in the experimental models used (that is, intact versus gonadectomized) or could be true species differences (see Trudeau *et al.*, 1991b for discussion). Nevertheless, the evidence in goldfish, carp and loach clearly indicates that the sex steroids have a predominant stimulatory effect on the pituitary responsiveness to sGnRH, cGnRH-II and several GnRH agonists.

Neuropeptide Y

The effect of NPY in stimulating GTH-II release from the pituitary is greatest in sexually mature female goldfish. The stimulatory effect of neuropeptide Y on GTH-II release is enhanced by testosterone and oestradiol, particularly in sexually regressed fish when endogenous steroid concentrations are lowest (Peng *et al.*, 1993b). Neuropeptide Y-induced GnRH release from preoptic area–anterior hypothalamic slices *in vitro* is also potentiated by implantation of testosterone and oestradiol *in vivo* (Peng *et al.*, 1993b). Moreover, testosterone and oestradiol stimulate expression of mRNA encoding neuropeptide Y in goldfish preoptic neurones (Peng *et al.*, 1994), indicating a brain site of action for the positive feedback effects of sex steroids, in addition to direct steroid effects on the pituitary.

Dopamine

Dopaminergic inhibition of GTH-II release, through a pituitary dopamine D_2 receptor, is greatest in sexually mature fish (Peter *et al.*, 1986). Testosterone and oestradiol enhance both pituitary dopamine turnover and dopamine inhibition of GTH-II release in sexually regressed goldfish (Trudeau *et al.*, 1993a). Moreover, pituitary dopamine turnover and the GTH-II release response to the dopamine D_2 antagonist, domperidone, increase during early gonadal development in the autumn (Trudeau *et al.*, 1993a; Sloley *et al.*, 1991), demonstrating a functional increase in gonadal negative feedback. Although studies have yet to be performed in goldfish, the trout oestrogen receptor has been cloned and sequenced, recombinant protein produced and specific antibodies against the hormone binding domain generated. The oestrogen receptor is localized in three principal neuroendocrine territories: the ventral telencephalon, the anterior preoptic region and the mediobasal hypothalamus; GnRH neurones in trout brain do not have the oestrogen receptor (Navas *et al.*, 1995), which is consistent with observations in mammals. However, the oestrogen receptor protein co-localizes with tyrosine hydroxylase in a subpopulation of preoptic neurones known to produce dopamine and innervate the trout pituitary (Linard *et al.*, 1995, 1996). Thus observations in goldfish and trout indicate that inhibitory dopaminergic neurones are directly responsive to oestradiol and are probably the principal mediators of negative feedback in fish.

Gamma-aminobutyric acid

GTH-II release stimulated by GABA *in vivo* varies seasonally and is modulated by sex steroids in goldfish (Kah *et al.*, 1992; Trudeau *et al.*, 1993b, c). GABA stimulates GTH-II release in the early stages of gonadal development but not in mature or sexually regressed animals. Increased serum GTH-II concentration in response to GABA is a true physiological signal; serum testosterone concentrations are also increased in goldfish treated with GVG (Sloley *et al.*, 1994). Testosterone increases, whereas oestradiol decreases, GABA-stimulated GTH-II release (Kah *et al.*, 1992; Trudeau *et al.*, 1993b). Thus, in the early stages of seasonal gonadal development, when testosterone concentrations in the blood begin to increase, goldfish respond to GABA, and testosterone enhances GABA action. With continuing gonadal development, serum concentrations of oestradiol also increase and appear to exert a negative feedback effect to reduce GABA responsiveness, possibly indicating an effect on GABA receptor binding. In addition to modulating GABA-stimulated GTH-II release, the gonadal steroids also affect GABA synthesis in both brain and pituitary; for example, testosterone and progesterone decrease and oestradiol increases pituitary GABA synthesis rates in sexually regressed goldfish (Trudeau *et al.*, 1993c). These data indicate that goldfish GABA neurones are exquisitely sensitive to changes in circulating sex steroid concentrations. It is not known whether GABA neurones in fish possess steroid receptors; however, they can act as transducers of complex endocrine signals to GnRH or dopamine neurones through variations in both neurotransmitter synthesis and action.

Gonadotrophic actions of growth hormone

In fish there is increasing evidence of interactions between GH and the reproductive axis. Growth hormone modulates gonadal steroidogenesis in addition to its effects on somatic growth (Le Gac *et al.*, 1993; Peter and Marchant, 1995). Singh *et al.* (1988) demonstrated that treatment with recombinant salmon GH prevents gonadal regression and stimulates testosterone and oestradiol production in hypophysectomized male and female killifish. In addition to direct effects, GH can potentiate GTH-II-stimulated steroidogenesis (Le Gac *et al.*, 1993). In seatrout, the mechanism of GH action is to stimulate ovarian aromatase activity, possibly via a cAMP-dependent pathway (Singh and Thomas, 1993). The ability of GH to stimulate oestradiol production may be part of a novel positive feedback system since, in goldfish, pituitary GH content (Zou *et al.*, 1996), and basal- and secretagogue-stimulated GH secretion (Trudeau *et al.*, 1992; Peng *et al.*, 1993b) are all enhanced by oestradiol treatment *in vivo*. The molecular basis for this stimulatory effect of oestradiol on GH production is unclear but is probably indirect, since fish GH genes do not contain consensus sequences for an oestrogen response element, and in goldfish oestradiol does not affect steady state pituitary mRNA encoding GH (Zou *et al.*, 1996). Growth is seasonally regulated in goldfish (Marchant and Peter, 1986), and maximal growth rates are found in the summer after the spring breeding period (Fig. 1). Progressively increasing GH secretion during gonadal development probably has two effects: (1) to act with GTH-II to stimulate gonadal development and steroidogenesis; and (2) to 'prime' the animal for post-spawning growth when water temperatures are increasing during the summer months. After spawning, when basal GTH-II concentrations have already decreased, GH secretion wanes but nevertheless remains high for several months (Fig. 1). Seasonal variation in somatic and gonadal development in teleosts is not synchronous, which may reflect differential release of GH and GTH-II. Indeed, there are both common and dissimilar neuroendocrine control mechanisms for release of these hormones from the pituitary (Fig. 5). This has been shown for dopamine, which stimulates GH via a D_1-like receptor but inhibits GTH-II release via a

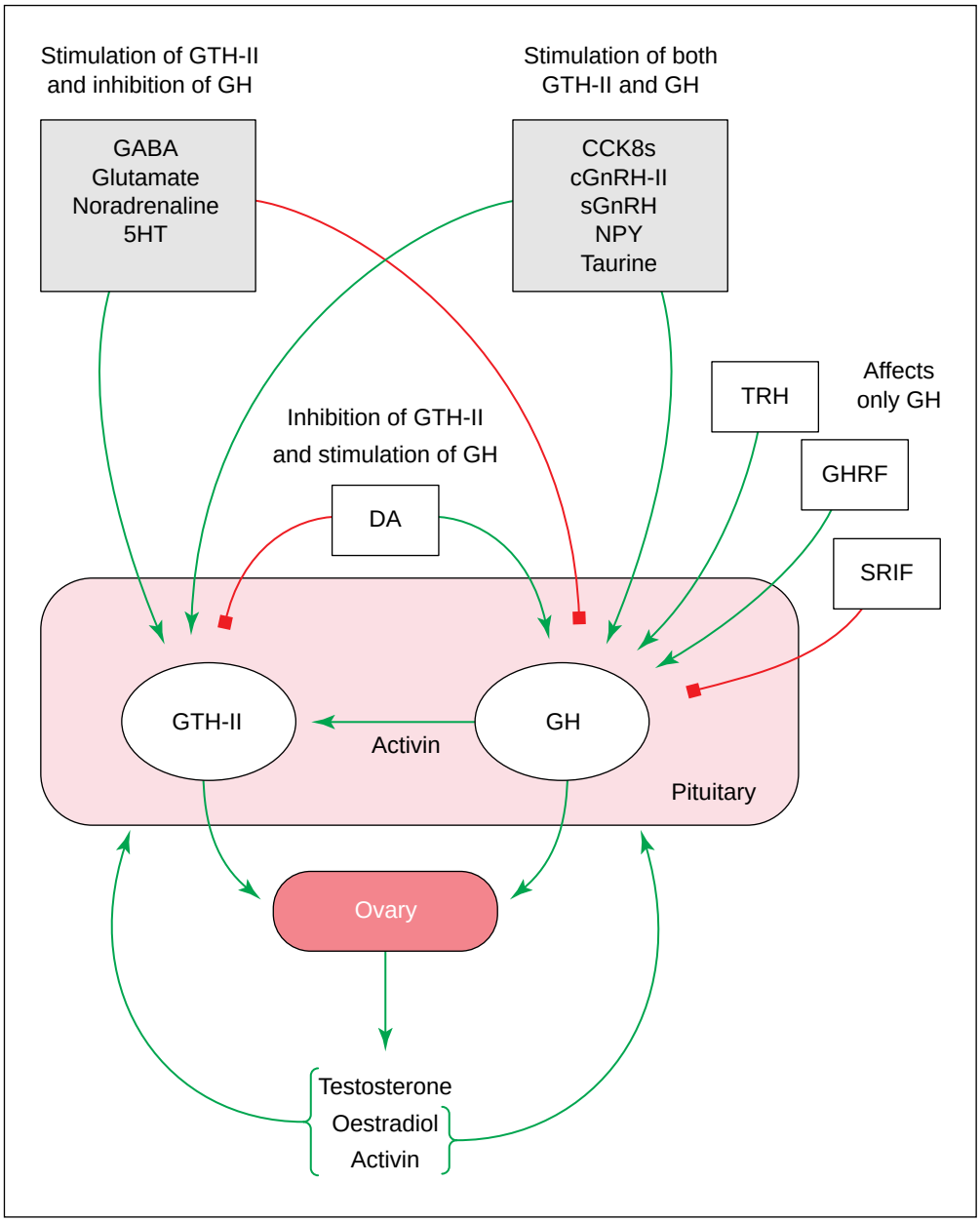


Fig. 5. Differential regulation of gonadotrophin II (GTH-II) and growth hormone (GH) release in the female goldfish. Neurohormones having similar effects on GTH-II or GH release are grouped functionally but this does not indicate that they are co-localized in the same neurones. For clarity, possible interactions and mechanisms of action of the various neurohormones are not shown. ➤ : stimulation; ⊣ : inhibition. cGnRH-II, chicken GnRH-II; CCK8s, sulfated cholecystokinin-8; DA, dopamine; GHRF, growth hormone-releasing factor; 5HT, 5-hydroxytryptamine; NPY, neuropeptide Y; sGnRH salmon GnRH; SRIF, somatostatin; TRH, thyrotrophin-releasing hormone.

D_2-like receptor (Wong *et al.*, 1993). In contrast, sGnRH and cGnRH-II (Marchant *et al.*, 1989), NPY (Peng *et al.*, 1993b), CCK8s (Himick *et al.*, 1993), taurine (Trudeau *et al.*, 1995) and activin (Ge *et al.*, 1992; Ge and Peter, 1994) stimulate both GH and GTH-II. Moreover, 5HT (Somoza and Peter, 1991), noradrenaline (Chang *et al.*, 1985), glutamate (Trudeau *et al.*, 1996) and GABA (Trudeau *et al.*, 1993c, unpublished) stimulate GTH-II release but inhibit GH release. Thyrotrophin-releasing hormone (TRH; Trudeau *et al.*, 1992) and growth hormone-releasing hormone (GHRH; Vaughan *et al.*, 1992) stimulate GH release, whereas somatostatin inhibits GH release (Marchant *et al.*, 1989); these three peptides have no effect on GTH-II release from the goldfish pituitary. This multifactorial and differential neuroendocrine control of GH and GTH-II release is probably the driving force behind seasonal variations in somatic and gonadal development in teleosts.

Integrated neuroendocrine control of seasonal gonadal development in goldfish

Circulating concentrations of GTH-II increase in the autumn and winter to stimulate the onset of ovarian redevelopment and the production of sex steroids (Fig. 6). Growth hormone concentrations are also increasing at this time but a clear role in seasonal gonadal development has not yet been defined. Testosterone, oestradiol and progesterone have positive feedback effects at the pituitary to enhance GnRH responsiveness and GTH-II release, and thus gonadal development is promoted by a positive feedback loop. Moreover, stimulation of NPY neurones and potentiation of NPY-stimulated GTH-II release by sex steroids must also contribute to positive feedback, as do non-steroidal factors such as activin and inhibin which both stimulate GTH-II release in goldfish. Gonadal steroids that enhance GnRH responsiveness do not affect basal GTH-II concentrations *in vivo* in gonad-intact goldfish (Trudeau *et al.*, 1991b, c, 1993a), indicating that there must also be activation of a potent negative feedback mechanism to control GTH-II release during gonadal development. Thus, concurrent activation of the inhibitory dopaminergic system keeps basal GTH-II release under tight negative feedback control (Trudeau *et al.*, 1993a), ensuring release of GTH-II only in response to the appropriate environmental or physiological stimuli. Given this apparent equilibrium between the positive feedback loop and the dopaminergic negative feedback system, why do basal GTH-II concentrations increase during seasonal gonadal development? It may be that in addition to enhanced GnRH action, multiple modulatory neuroendocrine systems (Fig. 2) are activated to allow GTH-II concentrations to increase during gonadal growth in the autumn and winter. One candidate modulatory neurotransmitter is GABA. This amino acid is a potent stimulator of GTH-II release in goldfish, reflecting its dual action to enhance GnRH release and to inhibit dopamine turnover. In mammals, GABA-regulated GnRH and LH release is characteristically acute, lasting for approximately 30–60 min (McCann and Rettori, 1988; Mitsushima *et al.*, 1994). In comparison, the GTH-II release response to GABA in goldfish is a rapid initial increase within 30 min, followed by a prolonged secretory response that can last from several hours to several days (Kah *et al.*, 1992; Trudeau *et al.*, 1993d; Sloley *et al.*, 1994). It is suggested that hypophysiotrophic GABA neurones in the goldfish brain act to convey important physiological and environmental information to the hypothalamic–pituitary complex, thus altering GTH-II synthesis and release to affect long term changes in gonadal function (Fig. 6). Gonadal steroids modulate many aspects of the neuroendocrine axis, including GABA synthesis and action, but the impact of annual variations in photoperiod or temperature on this system remains to be determined.

Superimposed on the seasonal increase in basal GTH-II concentrations is the surge release of GTH-II in male and female fish at the time of spawning. The preovulatory GTH-II surge is well described in goldfish, and is linked to co-ordinated responses to environmental cues (temperature, photoperiod and vegetation for egg deposition) and sex pheromones (Aida, 1988; Stacey *et al.*, 1994). Increased GnRH release and decreased dopaminergic activity are the principal mechanisms thought to induce surge release of GTH-II and ultimately ovulation and sperm release (See Peter *et al.*, 1991 for review). Understanding this GnRH and dopaminergic control of GTH-II release has led to the development of an effective spawning induction kit (injectable GnRH agonist with a dopamine antagonist) for fish aquaculture (Peter *et al.*, 1993). It has not been established whether the other neuroendocrine systems are also involved in the ovulatory GTH-II surge, but it is likely that many will be found to be important.

Information on the signals or neuroendocrine factors responsible for post-spawning regression of gonadal tissues is still lacking. Is it simply the loss of steroidogenic tissues after ovulation removing the positive feedback drive to neuroendocrine neurones and the pituitary? This is one plausible explanation since dopaminergic inhibition of GTH-II release is actually lowest in sexually regressed goldfish (Peter *et al.*, 1986; Sloley *et al.*, 1991; Trudeau *et al.*, 1993a). Are there other inhibitory neuroendocrine systems to be identified that mediate gonadal regression?

Conclusions

In fish, as in other vertebrates, GnRH is a major stimulator of reproduction, regulating the release of gonadotrophic hormones from the pituitary. However, multiple co-existing GnRH forms and variations in GnRH receptor signal transduction provide alternative control mechanisms in teleosts. The direct innervation of the proximal pars distalis in fish is the functional equivalent of the median eminence; multiple stimulatory inputs to the anterior pituitary are opposed by a potent inhibitory dopaminergic system. Studies of the multifactorial control of GTH-II raise the question of whether GnRHs are actually required if GTH-II release can be directly stimulated by other neurohormones in fish. Until recently, it was not possible to address this problem because of a lack of fish GnRH antagonists. However, fish GnRH antagonists have now been synthesized and characterized, and indeed, GnRH mediates both basal and stimulated GTH-II release in goldfish (Murthy *et al.*, 1993, 1994). Therefore, it can be proposed that GnRH is a key player in the neuroendocrine control of GTH-II release and many of the other stimulatory neurohormones may have more subtle modulatory roles. It must be emphasized that the physiological circumstances in which many of the stimulatory neuroendocrine neurones become active have not been established. Nevertheless, it is the balance between the stimulatory and inhibitory systems that ultimately determines the pattern of GTH-II release, and sex steroids have important roles in modulating neuroendocrine function. By using the gonad-intact goldfish model, positive and negative sex steroid feedback have been demonstrated in both sexes. Initially, the discovery that testosterone potentiated GnRH-induced GTH-II release (Trudeau *et al.*, 1991b) was particularly surprising, and contrasted with considerable data in mammals (Kalra and Kalra, 1983; Fink, 1988) and fish (Kobayashi and Stacey, 1990; Schulz *et al.*, 1993a) suggesting that testosterone only inhibited gonadotrophic hormone secretion. However, in these latter examples, gonadectomized animals were most often used. Therefore, it appears that in normal fish with functional gonads, both positive and negative feedback act concurrently to regulate GTH-II release finely. Growth hormone may also be part of a novel positive gonadal feedback system whereby GH enhances ovarian oestradiol production. In turn, oestradiol potentiates both

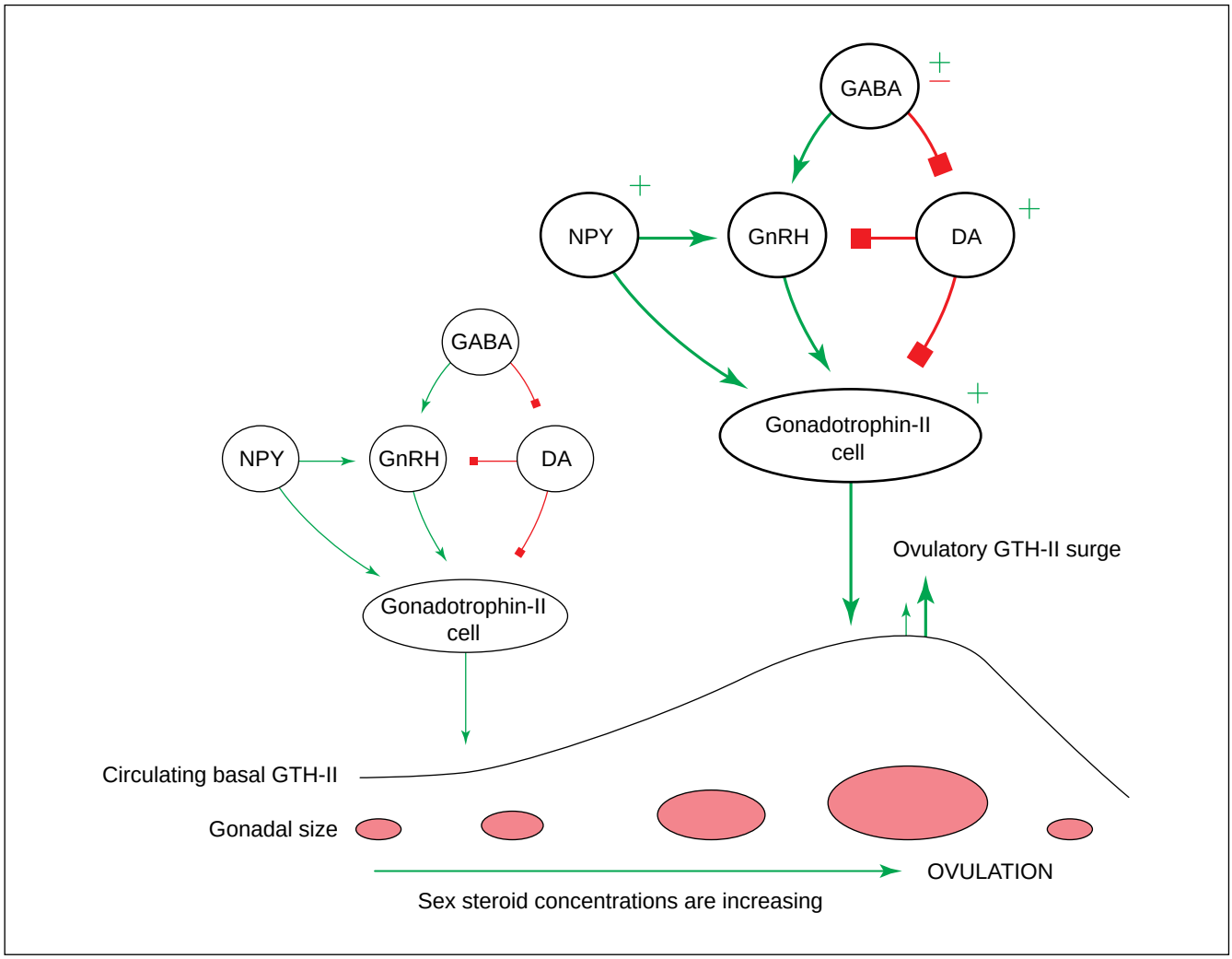


Fig. 6. Integrated neuroendocrine control of seasonal gonadal development in female goldfish. Stimulatory (➤) and inhibitory (■) interactions between GnRH, neuropeptide Y (NPY), dopamine (DA) and γ-aminobutyric acid (GABA) neurones are indicated. The increasing activities of these neuroendocrine systems in relation to seasonal gonadal development are shown by larger symbols. In goldfish, circulating basal gonadotrophin II (GTH-II) concentrations increase in the autumn to stimulate the onset of ovarian recrudescence and the production of sex steroids. Growth hormone (not shown) also has gonadotrophic actions in goldfish by enhancing ovarian oestradiol production. Testosterone and oestradiol have positive feedback effects at the pituitary to enhance GnRH responsiveness and GTH-II release, and in a feed-forward manner stimulate gonadal development. Pituitary GTH-II content and GnRH receptor binding increase in parallel with gonadal development. + indicates the known sites for a stimulatory effect of the sex steroids on a neurohormonal system; ± indicates that the sex steroids can either stimulate or inhibit GABA neuronal function. Stimulation of NPY gene expression and potentiation of NPY-stimulated GTH-II release by sex steroids also contributes to positive feedback. Sex steroids do not affect basal GTH-II concentrations *in vivo* in gonad-intact adults, indicating that there must also be activation of a negative feedback system to regulate GTH-II release. Thus, concurrent steroidal activation of the inhibitory dopaminergic system keeps GnRH and GTH-II release under tight negative feedback control. Given this apparent balance between positive feedback and negative feedback, how do basal GTH-II concentrations increase during gonadal development? It can be hypothesized that multiple stimulatory neuroendocrine systems must be activated to allow GTH-II concentrations to increase during gonadal development. One pivotal neurotransmitter is GABA which modulates both the stimulatory GnRH and inhibitory dopamine neurones. Surge release of GTH-II (↑; not to scale) at the time of spawning induces ovulation (see Aida, 1988; Peter *et al.*, 1991; Stacey *et al.*, 1994). The factors responsible for regression of the pituitary–gonadal axis after ovulation have not been identified.

GTH-II and GH release from the pituitary during gonadal development. A major finding in goldfish is that GABA is a prominent stimulator of GTH-II release, and plays a pivotal role by modulating both the GnRH and dopaminergic neuronal systems. Moreover, glutamate through activation of the NMDA receptor appears to play only a minor role in stimulating GTH-II release in fish. Thus, amino acid regulation of GTH-II release in fish differs from that in mammals, in which GABA is a major inhibitor and glutamate is a major stimulator of LH release. The multifactorial control of GTH-II synthesis and secretion is a complex and interactive process, and doubtless further differences between fish and other vertebrates await discovery.

Models are essential for a full understanding of neuroendocrine feedback loops. The adult goldfish as a member of one of the largest vertebrate families – the Cyprinidae (2000 species compared with 1000 in the Muridae; that is, rats and mice) – can be used as a key and powerful model for such studies, especially those involving positive neuroendocrine control of GTH release. Many neurohormones act both directly at the gonadotroph, and indirectly, through modulation of the GnRH neuronal system to regulate GTH-II release and seasonal reproductive cyclicity. The proposed model for the neuroendocrine control of GTH-II release is not definitive, as many other aspects of the goldfish neuroendocrine system remain to be investigated.

Much of the work reviewed was conducted in collaboration with Professor R.E. Peter (Edmonton); without his constant and continuing support these studies would not have been possible. Discussions and collaborations with many colleagues, especially Olivier Kah (Rennes), Duff Sloley (Edmonton), John Chang (Edmonton) and Makito Kobayashi (Tokyo) have also contributed to the ideas presented. This review was written following the encouragement of Professor P. Sharp (Edinburgh). Financial support from AHFMR (Canada) and the Wellcome Trust (UK) is also acknowledged with appreciation.

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