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## Chapter XI

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# Hypothalamic Regulation of Pituitary Gonadotropins

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## Abstract

Fish reproduction is regulated by the hypothalamus-pituitary-gonadal (HPG) axis. Within the HPG axis, gonadotropin-releasing hormone (GnRH) has been well recognized as the master molecule of reproduction, which regulates the synthesis and release of gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), from the pituitary. In recent years, new molecules such as kisspeptins and gonadotropin-inhibitory hormone (GnIH) in the HPG axis have become the focus of research in reproductive neuroendocrinology. Each of them is considered to play an important role in the control of the HPG axis, in particular in the positive and negative feedback actions. In this chapter, we summarize the old and new molecules in the HPG axis from anatomical and physiological points of view.

## Introduction

As in other vertebrates, the hypothalamus-pituitary-gonadal (HPG) axis predominantly regulates teleost reproduction. The hypothalamus receives a variety of internal and external environmental information and integrates it to regulate the pituitary (hypophysis). The hypothalamus contains many neuropeptides and neurotransmitters that stimulate pituitary gonadotropes (Table 1). Among them, gonadotropin-releasing hormone (GnRH) synthesized in a specific neuronal population of the hypothalamus has been known as the master molecule of reproduction to stimulate the synthesis and release of gonadotropins (GnHs; follicle-stimulating hormone, FSH, and luteinizing hormone, LH) in the pituitary.

**Table 1. Hypothalamic hormones and neurotransmitters that regulate gonadotropes**

	Hormone/ neurotransmitter	Fish species	Function	Reference	
Stimulatory	GnRH1	Blue gourami	Stimulation of FSH $\beta$ mRNA expression	(Levy and Degani, 2012)	
	GnRH2	Goldfish	Stimulation of LH release	(Chang et al., 1990)	
	GnRH3	Coho salmon	Stimulation of FSH and LH release	(Dickey and Swanson, 2000)	
		Tilapia	Stimulation of all GtH subunit mRNA expression	(Yaron et al., 2001)	
	GnIH (LPXRFa)	Masu salmon	Stimulation of LH, FSH and GH release	(Amano et al., 2006)	
		Grass puffer	Stimulation of LH and FSH mRNA expression	(Shahjahan et al., 2011)	
		Goldfish	Stimulation of LH $\beta$ mRNA expression	(Moussavi et al., 2012)	
	PQRFa	Hagfish	Stimulation of GtH $\beta$ mRNA expression	(Osugi et al., 2011)	
	26RFa	Goldfish	Increase of plasma LH levels	(Liu et al., 2009)	
	Kiss1	Sea bass	Increase of plasma LH levels	(Felip et al., 2009)	
		Goldfish	Stimulation of LH release	(Yang et al., 2010)	
	Kiss2	Zebrafish	Stimulation of FSH and LH mRNA expression	(Kitahashi et al., 2009)	
		Sea bass	Increase of plasma FSH and LH levels	(Felip et al., 2009)	
	Tachykinin (NKBa, NKF)	Zebrafish	Increase of plasma LH levels	(Biran et al., 2012)	
	Serotonin (5-HT)	Goldfish	Increase of plasma LH levels	(Somoza et al., 1988)	
		Atlantic croaker	Stimulation of LH release	(Khan and Thomas, 1992)	
	NPY	Goldfish	Stimulation of LH release	(Peng et al., 1990)	
			Common carp	Stimulation of LH release	(Breton et al., 1991)
			Rainbow trout	Stimulation of LH release	(Cerde-Reverter et al., 1999)
			Sea bass	Stimulation of LH release	(Cerde-Reverter et al., 1999)
Tilapia			Stimulation of LH $\beta$ and GtH $\alpha$ mRNA expression	(Yaron et al., 2001)	
PACAP		Goldfish	Stimulation of LH release	(Wong et al., 1998; Sawisky and Chang, 2005)	
		Tilapia	Stimulation of all GtH subunit mRNA expression	(Yaron et al., 2001)	
		Blue gourami	Stimulation of FSH $\beta$ mRNA expression	(Levy and Degani, 2012)	
Inhibitory	GnIH (LPXRFa)	Goldfish	Decrease of serum LH levels	(Moussavi et al., 2012; Zhang et al., 2010)	
			Inhibition of FSH $\beta$ and LH $\beta$ mRNA expression		
	Dopamine	Goldfish	Decrease of plasma LH levels	(Chang et al., 1983)	
Chinese loach			Decrease of plasma LH levels	(Lin et al., 1989)	

The release of GnRH and the sensitivity of gonadotropes (FSH and LH cells) to GnRH primarily determine the activity of the HPG axis. GtHs travel throughout the body with the bloodstream and stimulate maturation of the gonads (ovaries and testes) by binding to their respective receptors on them. Matured gonads secrete sex steroid hormones (estrogens and androgens), which negatively regulate the hypothalamus and the pituitary (long- and short-negative feedback loops). This closed-loop system maintains the homeostasis of the reproductive system. On the other hand, in certain situations such as the LH surge in females and the onset of puberty, sex steroids exert stimulatory effects on the hypothalamus and the pituitary (long- and short-positive feedback loops) to initiate the activation of the HPG axis.

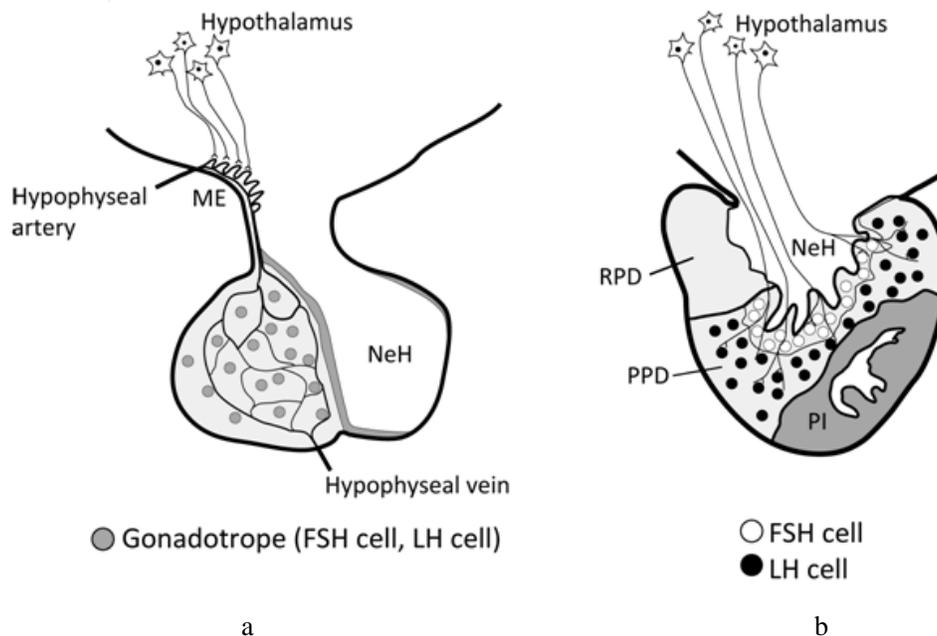
Although these mechanisms are very similar in teleosts and tetrapods, there are still significant differences in the roles of individual hypothalamic hormones and the way they are delivered to the pituitary as discussed in later sections. Since GnRH was identified as a neuropeptide that stimulates GtH secretion to play a pivotal role in regulating reproduction, this hypophysiotropic hormone has been recognized as the master molecule of reproduction in all vertebrates.

However, some aspects of the regulatory mechanism of reproduction, especially the sites of negative and positive feedback actions of sex steroids on GnRH secretion, remain unclear. In the last decade, several novel hypothalamic neuropeptides including kisspeptins and gonadotropin-inhibitory hormone (GnIH) have been identified as new players in the control of reproduction. Importantly, these novel neuropeptides fill the “missing link” of the negative and positive feedback actions of sex steroids in mammals. Therefore, the whole picture of the regulatory mechanism of reproduction is more evident now.

In this chapter, we summarize recent knowledge of the hypothalamic neuroendocrine mechanisms in the regulation of pituitary GtHs with specific emphasis on the comparison between teleosts and tetrapods.

## **Anatomical Characteristics of the Teleost Hypothalamus-Pituitary Connection**

The pituitary gland, which consists of the adenohypophysis and the neurohypophysis, is attached to the hypothalamus by a short stalk that contains neurosecretory fibers projecting from the brain to the pituitary in all vertebrates including fishes (Figure 1). The neurohypophysis is a posterior part of the pituitary, with a rich supply of neurosecretory fibers that release vasotocin and isotocin (vasopressin and oxytocin in mammals). On the other hand, the adenohypophysis, an anterior part of the pituitary, contains various pituitary endocrine cells secreting pituitary hormones, which include FSH, LH, thyroid-stimulating hormone (TSH), growth hormone (GH), prolactin (PRL), adrenocorticotrophic hormone (ACTH), and somatolactin (SL). The adenohypophysis is further divided into the pars distalis (rostral and proximal pars distalis) and the pars intermedia (Olivereau and Ball, 1964). The dorsal and ventral parts of the proximal pars distalis contain FSH and LH cells (Figure 1b), respectively (Zohar et al., 2010). The relatively segregated cell localization of hormone types to given pituitary regions compared to mammals is one of structural features of the fish pituitary.



ME, median eminence; NeH, neurohypophysis; PI, pars intermedia; PPD, proximal pars distalis; RPD, rostral pars distalis.

Figure 1. Innervations of hypophysiotropic hypothalamic neurons in mammals (A) and teleosts (B). Innervations of GnRH neurons are shown as an example. Within the adenohypophysis (anterior pituitary), the pars distalis and the pars intermedia are shaded with light and dark gray, respectively. In mammals, the axons of hypophysiotropic neurons terminate at the ME, and released hormones reach target cells via the blood circulation in the hypophyseal portal system. In teleosts, hypophysiotropic neurons directly innervate into the adenohypophysis (the rostral pars distalis and the proximal pars distalis) and terminate near to their target cells, which are located in a cluster manner.

Fish hypothalamus-pituitary connection also has a characteristic anatomical structure when compared to that of mammals. In fish, hypothalamic hormones that control pituitary functions are directly transported to the respective pituitary endocrine cells via neuronal fiber projections to the adenohypophysis (Figure 1b). Indeed, neuronal processes immunoreactive to various hypothalamic hormones project into the pituitary. These hypothalamic hormones are released from the nerve terminals to act directly on nearby target cells. On the other hand, in the mammalian HPG axis, neuronal fibers of the hypophysiotropic hormone cells terminate at the median eminence (ME), which is anatomically located just above the adenohypophysis, and release hormones into the hypophyseal portal system. Released hypothalamic hormones travel with the bloodstream to their target pituitary endocrine cells, which are scattered throughout the adenohypophysis (Figure 1a). Because hypothalamic hormones are delivered throughout the adenohypophysis via the blood circulation, the responsiveness of each pituitary endocrine cell to the hypophysiotropic hormones is determined by the expression of respective receptors in the target pituitary endocrine cells. Direct innervations from the hypothalamus to the adenohypophysis and the segregation of endocrine cells in the pituitary makes teleost a useful model to understand hypothalamic control of the pituitary endocrine cells (Parhar and Iwata, 1994).

In fact, there is good association between the distribution of hypothalamic neuronal fibers and the endocrine target cells in the adenohypophysis of teleosts. For example, fiber terminals of GnRH neurons are distributed in the proximal pars distalis, where LH and FSH cells are located, which justifies the role of GnRH in GtH secretion. Neurons synthesizing several neuropeptides and neurotransmitters, particularly GnRH (Parhar, 1997), GABA (Kah et al., 1987a), neuropeptide Y (Batten et al., 1990), dopamine (Kah et al., 1984), and PACAP (Wong et al., 1998) have been identified to project their fibers to the pars distalis to modulate the release of GtH and other pituitary hormones (Table 1).

Among brain regions, two hypophysiotropic nuclei, namely the nucleus preopticus (NPO) and the nucleus lateralis tuberis (NLT), have been known to control teleostean adenohypophyseal functions. In most teleosts, the NPO and the NLT comprise the major neuronal systems that innervate the pituitary to regulate different endocrine cells (Fryer and Maler, 1981; Peter, 1986). However, some extra hypothalamic areas, especially from the olfactory system and the telencephalon, have also been identified to send neuronal projections to the pituitary (Anglade et al., 1993; Chiba et al., 1996; Rama Krishna et al., 1992). In the POA-hypothalamus, the anterior and posterior subdivisions of the nucleus preopticus periventricularis (NPP) have been identified as the regions that contain a variety of peptide hormones and neurotransmitters, including GnRH (Parhar, 1997) and dopamine (Kah et al., 1984), which are involved in the control of GtH secretion in the pituitary.

## **Hypothalamic Hormones Regulating GtH Secretion**

### **Gonadotropin-Releasing Hormones (GnRHs)**

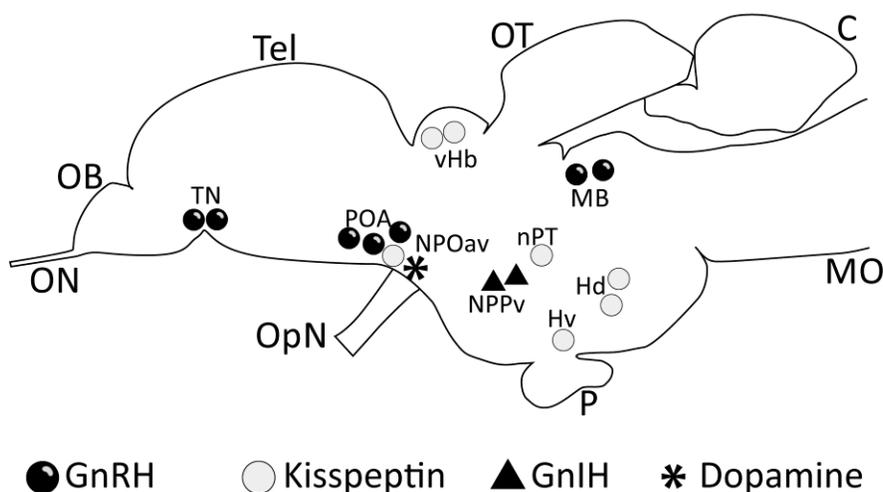
GnRH was first identified from the ovine and porcine hypothalamus as a peptide hormone that stimulates the release of LH from the adenohypophysis (Amoss et al., 1971; Matsuo et al., 1971), and was called luteinizing hormone-releasing hormone (LHRH). It was identified as a decapeptide with the primary structure of pEHWSYGLRPG-NH<sub>2</sub>. Findings that this hypophysiotropic hormone also stimulates the secretion of FSH changed its name to gonadotropin-releasing hormone, GnRH. The first fish GnRH was identified from the salmon by Sherwood and coworkers (Sherwood et al., 1983). To date, 15 forms of GnRH with different amino acid sequences have been identified in vertebrates, including ten teleost-specific forms like salmon, seabream, whitefish, medaka, catfish, herring, dogfish, lamprey I, II and III (Adams et al., 2002; Carolsfeld et al., 2000; Jimenez-Linan et al., 1997; Kavanaugh et al., 2008; Lovejoy et al., 1992; Matsuo et al., 1971; Miyamoto et al., 1982; Miyamoto et al., 1984; Ngamvongchon et al., 1992a; Okubo et al., 2000; Powell et al., 1994; Sherwood et al., 1983; Sower et al., 1993; Yoo et al., 2000). It now appears that most vertebrate species possess two, and some teleost species have three, GnRH forms (Parhar, 1997; Sherwood et al., 1997), although rodents, bovine, and sheep are known to possess only one functional GnRH (Millar, 2003; Morgan et al., 2006). Fish species possess not only the most diverged primary GnRH structure, with 12 in total (10 fish forms, mammalian GnRH, and chicken GnRH-II), but also possess up to 5 GnRH receptor (GnRH-R) variants in some species (Jodo

et al., 2003; Kah et al., 2007). Fish GnRH is therefore of interest from an evolutionary point of view.

It has been reported that GnRH and GnRH-R are also expressed in peripheral tissues including the gonads (Lin et al., 2010; Madigou et al., 2002; Pati and Habibi, 1998; Uzbekova et al., 2002; Yu et al., 1998). This gonadal GnRH/GnRH-R system is considered to act in an autocrine or paracrine manner to regulate gonadal steroidogenesis and oocyte maturation (Andreu-Vieyra et al., 2005; Pati and Habibi, 2000). However, in this chapter, we focus only on the central roles of GnRH in the brain.

While GnRH was traditionally named after the species in which it was identified, the same form also exists in other species; salmon GnRH also exists in the herring, goldfish, medaka, seabream, and the tilapia.

Therefore, the current nomenclature uses GnRH1, GnRH2 and GnRH3 based on the phylogenetic sequence analysis and respective location of expression. GnRH1 is the most variable form among the three, and is generally expressed in the ventral forebrain-preoptic area (POA)-basal hypothalamus. GnRH2 is the most evolutionarily conserved GnRH form among vertebrates and expressed in the midbrain. The third type GnRH3 is present in the caudal-most olfactory bulb (Figure 2) along the terminal nerve in certain fish species (Okubo and Nagahama, 2008). GnRH1, of which neurons are located in the POA-basal hypothalamus and send neuronal fibers directly into the adenohypophysis (teleosts) or to the ME (other vertebrates), is considered to be a hypophysiotropic form. In most fish species, GnRH1 neurons are the major source of GnRH fiber projections into the pituitary.



C, cerebellum; Hd, dorsal zone of periventricular hypothalamus; Hv, ventral zone of periventricular hypothalamus; MB, midbrain; MO, medulla oblongata; NPOav, Nucleus preopticus anteroventralis; NPPv, nucleus posterioris periventricularis; nPT, posterior tuberal nucleus; OB, olfactory bulb; ON, olfactory nerve; OpN, optic nerve; OT, optic tectum; P, pituitary; POA, preoptic area; Tel, telencephalon; TN, terminal nerve; vHb, ventral habenula.

Figure 2. A schematic drawing of representative localization of GnRH (black circle), kisspeptin (white circle), GnIH (triangle), and dopamine (asterisk) neurons in a sagittal section of the teleost brain. GnRH neurons in the POA, GnIH neurons in the NPPv, and dopaminergic neurons in the NPOav are shown to innervate the pituitary, while the kisspeptin neuronal population(s) projecting into the pituitary need to be identified.

As in mammals, the LH-releasing role of GnRH has been established in fish species (Trudeau, 1997; Yaron et al., 2003). On the other hand, the lack of quantification method of FSH peptide in most fish species limits information about GnRH effects on FSH release. Some studies in salmonids, however, show that, as in mammals, GnRH also stimulates FSH release from the fish pituitary (Ando et al., 2004; Breton et al., 1998; Dickey and Swanson, 2000; Kawauchi et al., 1989). From these studies, it appears that GnRH1 stimulates the release of FSH and LH during early and late maturational stages, respectively.

Quantitative gene expression analyses of GtH subunits showed the role of GnRH in the regulation of GtH synthesis is dependent on the maturational stage and the fish species (Yaron et al., 2003). For example, in maturing sockeye salmon GnRH analog (GnRH<sub>a</sub>) significantly increases the amounts of LH $\beta$  and GtH $\alpha$  subunit mRNAs in both sexes, while there is no effect on FSH $\beta$  subunit mRNA (Kitahashi et al., 1998a).

Similar stimulatory effect on LH subunit genes is observed in reproductively quiescent sea bass *Dicentrarchus labrax* (Mateos et al., 2002). On the other hand, in maturing striped bass and common carp the stimulatory effect of GnRH on gene expression is seen in both FSH and LH subunits in males whereas only LH synthesis is stimulated by GnRH in females (Hassin i, 1998; Kandel-Kfir et al., 2002). *In vitro* studies using a primary culture of pituitary cells from different maturational stages of fish further confirmed that GnRH has direct effects on the synthesis and release of FSH and LH in a maturational stage-dependent manner in different species (Ando and Urano, 2005).

GnRH1 neurons also project into other brain areas including the olfactory bulbs, olfactory nerve, optic tectum, and the spinal cord (Parhar, 1997). The extra-hypothalamic fiber projections of GnRH1 suggest its role in other reproductive functions such as sexual behavior or non-reproductive behavior.

The functions of GnRH2 and GnRH3 are debatable due to limited evidence. Generally, GnRH2 and GnRH3 fibers are observed throughout the brain, but not in the pituitary. Thus, they are thought to have neuromodulatory functions rather than hypophysiotropic functions (Sherwood et al., 1997). This is supported by the spontaneous regular pacemaker activities of GnRH2 and GnRH3 neurons compared to the episodic firing of GnRH1 neurons observed in teleosts (Oka, 2009). In fact, administration of GnRH2 markedly reduced food intake in the goldfish (Matsuda et al., 2008) through an inhibitory effect on orexin neurons (Hoskins et al., 2008). The involvement of GnRH2 in feeding has also been reported in a mammalian species, the musk shrew *Suncus murinus* (Kauffman and Rissman, 2004; Kauffman and Rissman, 2006), suggesting its role in the control of feeding is evolutionally conserved. In addition, a recent study in the sea bass suggested GnRH2 stimulates melatonin secretion from the pineal gland (Servili et al., 2010).

GnRH3 has been suggested to be involved in the control of reproductive behaviors. Immunoneutralization of GnRH3 using a specific antibody significantly decreases aggressive behavior and nest-building ability in the male Nile tilapia (Ogawa et al., 2006). Lesions of GnRH3 neurons cause impairment of nest-building in male dwarf gourami (Yamamoto et al., 1997). Administration of GnRH<sub>a</sub> shortens the duration of homing migration of the pre-spawning lacustrine sockeye salmon, *Oncorhynchus nerka*, which further provides evidence of its role in stimulating reproductive behavior (Kitahashi et al., 1998b; Sato et al., 1997).

In spite of the similar neuronal localization pattern and function of each GnRH form in different fish species, there are slight variations in the GnRH peptides detected in the pituitary

among fish species. For example, GnRH2 is detected in the pituitary of cyprinids (Steven et al., 2003; Yu et al., 1988) and catfish (Ngamvongchon et al., 1992b; Schulz et al., 1993). GnRH3 neurons are also shown to project into the pituitary of the sea bass, but significantly less than GnRH1 neurons (Gonzalez-Martinez et al., 2002a). However the significance of the species differences in pituitary innervation of GnRH neurons is still not clear.

In some fish species including most salmonids and zebrafish, the GnRH1 gene has been lost during evolution. In such species, GnRH3 seems to double as GnRH1 in terms of its function and neuronal localization (Abraham et al., 2009; Amano et al., 1997): i.e. GnRH3 neurons are located in the olfactory bulb, ventral telencephalon, POA, and the ventromedial hypothalamus, and send fibers into the pituitary.

## Kisspeptins

Kisspeptin is a member of the RFamide family of peptides, encoded by the *KISS1* gene in humans (Kotani et al., 2001). The core 10-amino acid sequence (Kiss1-10) is common among endogenous mature peptides derived from a common precursor peptide. This core sequence is highly conserved during evolution and shows potent biological activity (Bilban et al., 2004). Its important role in reproductive control emerged in 2003 when two independent groups reported inactivating mutations in kisspeptin receptor (*kiss1r*, G-protein coupled receptor 54, GPR54) result in idiopathic hypogonadotropic hypogonadism in humans and mice (de Roux et al., 2003; Seminara et al., 2003). *Kiss1r* knockout mice also exhibit the failure of pubertal onset (Lapatto et al., 2007; Seminara et al., 2003). Furthermore, similar phenotypes were demonstrated by *Kiss1* knockout in mice, confirming the role of kisspeptin/*kiss1r* in the onset of mammalian puberty (d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007). Kisspeptin administration induces FSH and LH release solely through the activation of GnRH1 release, because treatment with a GnRH antagonist completely blocks the effects of kisspeptin in rodents (Navarro et al., 2005a; Navarro et al., 2005b). Extensive studies revealed that kisspeptin fibers project to GnRH1 neuronal bodies and fiber terminals to regulate GnRH1 secretion at the POA and ME levels, respectively (Parhar et al., 2012).

In rodents, kisspeptin neurons are localized in the arcuate nucleus (Arc) and the anteroventral periventricular nucleus (AVPV) (Clarkson and Herbison, 2006). The two kisspeptin neuronal populations are differently regulated by sex steroids. In the Arc, kisspeptin synthesis is inhibited by sex steroids, while it is stimulated in the AVPV (Smith et al., 2005a; Smith et al., 2005b). Thus, in mammals, kisspeptin neurons in the Arc and the AVPV are considered to mediate the negative and positive feedback actions of sex steroids, respectively.

In teleosts, similar to GnRH, kisspeptins present in multiple forms and multi-functional in one species. The first identification of the cDNAs for two kisspeptin genes, *kiss1* and *kiss2*, was reported in the medaka and the zebrafish (Kitahashi et al., 2009). cDNAs for the two kisspeptin genes have also been cloned in the chub mackerel (Selvaraj et al., 2010), sea bass (Felip et al., 2009), and the goldfish (Li et al., 2009).

Genome comparison suggests that *kiss1* and *kiss2* are paralogs resultant from a whole genome duplication event during vertebrate evolution. Indeed, most vertebrates possess two forms of kisspeptin whereas *kiss2*-homologous sequences are absent in placental mammals.

The absence of *kiss2* could be due to the loss of the *kiss2* gene in this lineage during evolution, because the genomic regions syntenic to where the *kiss2* gene is located in teleost still exist in the mammalian genomes (Kitahashi et al., 2009). In parallel to the multiple kisspeptin forms, there are multiple kisspeptin receptors in teleosts. In the zebrafish, the core sequences of two kisspeptins (Kiss1-10 and Kiss2-10) showed the highest potency for kiss1 receptor (Kiss1R, also called GPR54-1) and kiss2 receptor (Kiss2R, also called GPR54-2), respectively (Lee et al., 2009).

Systemic kisspeptin administrations (Kiss1-10 and Kiss2-10) stimulate the synthesis and release of FSH and LH and gonadal maturation in fish as in mammals. However, the effect of Kiss1-10 and Kiss2-10 in the gonadotrope control varies among species. In sexually mature female zebrafish, intraperitoneal injections of Kiss2-10 but not Kiss1-10 significantly increased the amount of FSH $\beta$  and LH $\beta$  mRNAs (Kitahashi et al., 2009). The stronger effect of Kiss2-10 compared to Kiss1-10 was also observed in the release of FSH and LH in prepubertal sea bass (Felip et al., 2009). A similar trend of the effects of kisspeptins was observed in the stimulatory effect on gonadal maturation in female sea bass and striped bass (Beck et al., 2012).

On the contrary, in female spotted grouper, an intraperitoneal Kiss2-10 injection stimulated the mRNA expression of FSH $\beta$  rather than LH $\beta$  (Shi et al., 2010). Furthermore, in the goldfish, an intraperitoneal Kiss1-10 injection, but not Kiss2-10, stimulated LH release in sexually mature female (Li et al., 2009). These results indicate the role of *kiss1* and *kiss2* in the regulation of GtH is species specific. However, it should be noted that longer kisspeptins, Kiss1-15 and Kiss2-12, are proposed as the endogenous kisspeptin forms in teleost species (Lee et al., 2009).

The anatomical localizations of kisspeptin neurons in the fish brain have been examined by *in situ* hybridization studies. These studies show *kiss1*-expressing neurons in the ventral habenula (vHb) and *kiss2*-expressing neurons in the dorsal zone of the periventricular hypothalamus (Hd; also designated as the nucleus recessus lateralis, nRL) in the zebrafish, medaka, goldfish, and the sea bass (Figure 2; Escobar et al., 2012; Kanda et al., 2012; Kitahashi et al., 2009). *Kiss1*-expressing neuronal populations have also been localized in the nucleus ventral tuberis (nVT) and the nucleus posterior is periventricularis (NPPv) in the medaka (Figure 2; Kanda et al., 2008; Kitahashi et al., 2009).

Interestingly, in the zebrafish and goldfish brains, the *kiss1* gene is not expressed in these hypothalamic neuronal populations. Instead, the *kiss2* gene is expressed in the nVT and NPPv kisspeptin neurons in the goldfish and the zebrafish, respectively (Kanda et al., 2012; Kitahashi et al., 2009; Servili et al., 2011). In addition, there is another *kiss2*-expressing neuronal population in the POA of the zebrafish and goldfish (Kanda et al., 2012; Servili et al., 2011). The variation in the kisspeptin form expressed in hypothalamic nuclei and the different effects of systemic administrations of Kiss1-10 and Kiss2-10 in different fish species raise the possibility that kisspeptin functions diverged during teleost evolution. Similar to the GnRH/ GnRH-R system, kisspeptins and kisspeptin receptors are also expressed in several peripheral tissues including the gonads, suggesting additional local actions of kisspeptins (Biran et al., 2008; Kitahashi et al., 2009; Shahjahan et al., 2010b; Yang et al., 2010).

Fiber projections of kisspeptin neurons in the brain provide important information to understand the endogenous roles of the two kisspeptins in neuroendocrine function. However, information on kisspeptin fiber projections in fish species had been limited by the lack of

specific antibodies for fish kisspeptin forms. Recent efforts to develop specific fish kisspeptin antisera and the application of a neuronal tracer revealed that the habenula kiss1 neurons send fibers only to the ventral part of the interpeduncular nucleus, IPN (Ogawa et al., 2012; Servili et al., 2011).

In addition, the expression of Kiss1 receptor is observed only in the habenula and the ventral IPN in the zebrafish (Ogawa et al., 2012). The habenula kiss1 system is thus implicated in the modulation of serotonergic system rather than HPG axis regulation in the zebrafish (Ogawa et al., 2012). On the other hand, Kiss2 fibers are widely distributed in the zebrafish forebrain and midbrain (Servili et al., 2011).

The fact that all three GnRH neuron types express kisspeptin receptors in the Nile tilapia (Parhar et al., 2004) suggests that the role of Kiss2 neurons in the regulation of the HPG axis is via the activation of the GnRH systems.

## Gonadotropin-Inhibitory Hormone (GnIH)

Gonadotropin-inhibitory hormone (GnIH) was found in the Japanese quail in 2000 as a hypothalamic neuropeptide that inhibits GtH release from the pituitary (Tsutsui et al., 2000). GnIH was identified as a dodecapeptide SIKPSAYLPLRF-NH<sub>2</sub>, and classified as a member of the RFamide family of peptides due to its C-terminal RF-NH<sub>2</sub> motif. GnIH is the first hypothalamic neuropeptide identified as an inhibitor of GtH release in vertebrates. In avian species, GnIH expressed in the paraventricular nucleus (PVN) inhibits the HPG axis at the level of GnRH1 neuronal somata and fiber terminals as well as its direct action on the pituitary gonadotropes (Tsutsui et al., 2012). In the last decade, GnIH and its orthologs have been identified in many vertebrates including birds, fishes, and mammals. As in avian species, mammalian GnIH (RFamide-related peptide-3, RFRP-3) suppresses GtH secretion via the inhibition of GnRH1 neurons, while its direct action on the pituitary is controversial in mammals (Tsutsui et al., 2012).

GnIH has been identified in fish species including the goldfish, zebrafish, medaka, *Takifugu*, grass puffer, and the sea lamprey (Osugi et al., 2006; Sawada et al., 2002; Zhang et al., 2010). Fish GnIH is also referred to as LPXRFa after the sequence of C-terminal motif of its mature peptide. The neurons expressing fish GnIH are localized in the NPPv of the goldfish and the sockeye salmon (Figure 2; Amano et al., 2006; Sawada et al., 2002). As in birds and mammals, fish GnIH neuronal fiber projections are observed in a wide range of brain regions, which include the area where GnRH1 neurons are located. In addition, some GnIH fibers also project to the pituitary in both species (Amano et al., 2006; Sawada et al., 2002), suggesting evolutionally conserved GnIH functions in teleosts.

The physiological function of GnIH in teleosts is complex. As in birds and mammals, intraperitoneal injections of the goldfish and zebrafish GnIH peptides reduce plasma LH levels in adult goldfish (Moussavi et al., 2012; Zhang et al., 2010). However, the inhibitory effect of GnIH injections is not observed in the late recrudescence stage (Moussavi et al., 2012).

In addition, a stimulatory effect of goldfish GnIH peptide on LH release is observed in cultured goldfish and salmon pituitaries (Amano et al., 2006; Moussavi et al., 2012). Furthermore, injections of GnIH drastically increase the levels of FSH and LH mRNAs in the

goldfish pituitary in a reproductive stage-dependent manner (Moussavi et al., 2012). *In vitro* studies also show that the goldfish GnIH peptide stimulates GtH subunit gene expression in the goldfish and the grass puffer with clear seasonal differences (Moussavi et al., 2012; Shahjahan et al., 2011). These findings indicate that, in teleosts, the physiological effect of GnIH on the HPG axis differs between GtH synthesis and release and depends on the reproductive stage. To our knowledge, interaction between GnIH and GnRH neurons in teleosts has yet to be reported, while close appositions between GnIH fibers and GnRH1 neuronal somata are evident in birds and mammals (Soga et al., 2010; Tobari et al., 2010; Ubuka et al., 2012; Ubuka et al., 2009). Further studies, especially using other seasonal/non-seasonal breeders are needed, which will provide clearer answers regarding the roles of GnIH in the control of the HPG axis.

## Dopamine

Dopamine is a monoamine neurotransmitter that plays a variety of functions in vertebrates. Dopaminergic neuronal distribution in the fish brain has been extensively studied. There are many dopaminergic neuronal populations and wide-spread fiber projections in the brain (Panula et al., 2010). Receptors for dopamine are classified into two major classes that activate (D1-like) or inhibit (D2-like) an enzyme adenylate cyclase (Cardinaud et al., 1998).

Dopamine has an inhibitory role in GtH release in many teleosts (Peter et al., 1986). This inhibitory action has been suggested to be a direct action because dopamine receptors are expressed in gonadotropes (Chang and Peter, 1983). A group of dopaminergic neurons localized in the nucleus preopticus anteroventralis (NPOav) of the ventral preoptic area directly innervate pituitary gonadotropes in the goldfish (Kah I, 1987b) and the European eel (Figure 2; Sébert et al., 2008). Using specific agonists and antagonists *in vivo* and *in vitro*, it was shown that dopamine inhibits GtH release directly through the D2-like receptors expressed in gonadotropes. Studies showed that dopamine inhibits spontaneous and GnRH-stimulated GtH release in the goldfish (Chang and Peter, 1983; Chang et al., 1990), catfish (de Leeuw et al., 1986), Chinese loach (Lin et al., 1989), tilapia (Levavi-Sivan et al., 1995) and the gray mullet (Aizen et al., 2005). In the goldfish, dopamine also acts on GnRH neurons to inhibit GnRH release (Trudeau, 1997). These results indicate that dopaminergic inhibition plays an important role in the control of teleost reproduction. However, the inhibitory role of dopamine in GtH release is not dominant in some fish species (Copeland and Thomas, 1989), suggesting that physiological importance and roles of the dopaminergic inhibition is species-specific in teleosts.

## Ontogeny of the GnRH and Kisspeptin Systems

GnRH neurons are known for their migration during embryonic development. In fish, GnRH1 neurons originate in the olfactory placode as in mammals and migrate to their final destination in the POA (Parhar, 2002). In the European sea bass, GnRH1 immunoreactivities start to appear at the olfactory placode on 26 days after hatching (dah). These neurons migrate along the olfactory tract, and are positioned at the POA and the hypothalamus at 45 and 60

dah, respectively (Gonzalez-Martinez et al., 2004; Gonzalez-Martinez et al., 2002b). The migration of GnRH1 neurons during early development, from the olfactory region to the POA, is crucial for the development of the reproductive system in all vertebrates. In patients with Kallmann's syndrome (KS), GnRH1 neurons fail to migrate due to mutations in a gene for anosmin-1, which is responsible for migration of certain nerve cell precursors during embryogenesis, and are trapped at the place where they are originated (Schwanzel-Fukuda et al., 1989; Wray et al., 1989). As a result, patients with KS exhibit hypogonadotropic hypogonadism together with anosmia (partial or complete loss of olfaction) (Naftolin et al., 1971). GnRH3 neurons also originate in the olfactory placode, but at a relatively early stage, around 7 dah, and migrate to the olfactory bulb on 10 dah in the European sea bass, appearing in the ventral telencephalon and the POA from days 15 and 45, respectively (Gonzalez-Martinez et al., 2004; Gonzalez-Martinez et al., 2002b).

In salmonids, immunoreactive GnRH neurons are first detected at 19 day after fertilization (daf) in the olfactory placode, appear in the olfactory nerve by 30 daf, in the olfactory bulb by 51 daf, and appear in the ventral telencephalon and the POA by 462 daf (Parhar et al., 1995). In a fast-developing zebrafish embryo, GnRH3 neurons start appearing in the olfactory placode around 26 hours after fertilization and migrate to the olfactory bulb, POA, and caudal hypothalamus by 4-5 daf (Abraham et al., 2008). Laser ablation of GFP-labeled GnRH3 neurons in the olfactory placode at an early stage of embryonic development completely diminished the number of GnRH3 neurons in the adult stage, indicating all populations of zebrafish GnRH3 neurons originate from the olfactory region (Abraham et al., 2010). On the other hand, GnRH2 neurons originate in the germinal zone of the third ventricle, at a diencephalic/mesencephalic transitional area (Parhar et al., 1996; White and Fernald, 1998). GnRH2 neurons start to be observed on 4 dah in the European sea bass during development (Gonzalez-Martinez et al., 2004; Gonzalez-Martinez et al., 2002b). The earliest expression of the midbrain GnRH2 among the three GnRH forms is also observed in other vertebrate species (Muske and Moore, 1990; White and Fernald, 1998), suggesting an important role of this evolutionally-conserved GnRH form in ontogenesis.

In terms of the roles of each GnRH form during development, the involvement of the GnRH neuronal system(s) in the onset of puberty is well documented in teleosts. Among the three GnRH forms, only hypothalamic GnRH (GnRH1) mRNA and peptide levels show coincident increase with gonadal development (Holland et al., 2001; Okuzawa et al., 2003; Parhar et al., 1996; Senthilkumaran et al., 1999; Shahjahan et al., 2010a), indicating its role in the onset of puberty in fish. On the other hand, during the pubertal stage, three forms of GnRH mRNA expression are significantly high, whereas only GnRH1 shows an increase at the latter stage during the reproduction in grey mullet (Nocillado et al., 2007). These results indicate that, in the species expressing three GnRH forms, GnRH1 has the gonadotropic role, while GnRH2 and GnRH3 have important roles in modulating reproductive behavior (Parhar et al., 2003a; Uchida et al., 2005).

In the last few years, considerable developments have been made in the study of puberty in mammals. It has been demonstrated that kisspeptin and kisspeptin receptor (kissR) play a crucial role in reproduction, mainly by stimulating GnRH release (Messenger, 2005), therefore acting as a gatekeeper for puberty.

In the zebrafish, the mRNA levels of kisspeptins (Kiss1 and Kiss2) are increased significantly at the start of the pubertal phase together with hypothalamic GnRH (GnRH3) mRNAs (Kitahashi et al., 2009). The levels of kissR mRNAs in the brain, which are

expressed in GnRH neurons (Parhar et al., 2004), are also higher at the start of puberty compared to the pre- or post-pubertal stages in the cobia (Mohamed et al., 2007), grey mullet (Nocillado et al., 2007), fathead minnow (Filby et al., 2008), and the tilapia (Martinez-Chavez et al., 2008). These results indicate that fish kisspeptins most likely control the onset of puberty by stimulating GnRH release, as in mammals (Dungan et al., 2006; Seminara, 2005).

## Ontogeny of the Pituitary Gonadotropes

Pituitary GtHs, FSH and LH, directly control gonadal development and reproduction in fish (Lubzens et al., 2010; Yaron et al., 2003). In multiple-spawning fish species, differential roles of FSH and LH in the control of reproductive cycles have been established. In general, FSH is predominant during early gonadal recrudescence and gametogenesis, whereas LH plays a major role in the regulation of final gonadal maturation, ovulation, and spermiation (Swanson et al., 1991; Yaron et al., 2003).

On the other hand, ontogeny studies of FSH and LH cells in the pituitary have suggested additional GtH roles in sex differentiation and pubertal onset. Immunoreactivities of both FSH and LH appear simultaneously in the pituitary; on 14 daf in the Nile tilapia and 51 daf in the sockeye salmon (Parhar et al., 2003b). The pituitary contents of FSH protein and mRNA are much higher than those of LH during early puberty in the masu salmon (Amano et al., 1993) and rainbow trout (Gomez et al., 1999). The earlier appearance of FSH cells compared to LH cells in the pituitary during development commonly occurs in teleost species (Gomez et al., 1999; Guzman et al., 2009; Hassin et al., 1999; Hassin et al., 2000; Moles et al., 2007; Parhar et al., 2003b; Wong et al., 2004). This suggests a role for FSH in sex differentiation and the early stages of gonadal development, whereas LH may play a role mainly in the pubertal stage. However, in some fish species such as the African catfish, pejerrey, and the ricefield eel, LH cells appear earlier than FSH cells (Miranda et al., 2001; Schulz et al., 1997; Wu et al., 2012), suggesting species-specificity in the respective roles of FSH and LH in early gonadal differentiation and development. Immunoreactivities against GnRH receptors (GnRH-R type IA and IB) are first observed in the pituitary on 15 daf in the Nile tilapia (Parhar et al., 2003b). In this species, which undergo sexual differentiation and gonadal steroidogenesis at around 25 daf, immunoreactivity for GnRH-R type IB in the pituitary is decreased at the time of sexual differentiation, while the expression of GnRH-R type IA is significantly increased (Parhar et al., 2002). The presence of GnRH-Rs type IA and IB in gonadotropes suggests that they mediate the different effects of GnRH on the synthesis/release of GtHs from the pituitary. Interestingly, the time of sexual differentiation in the tilapia, salmonids, and the eel coincides with the development of GnRH fibers in the pituitary (Parhar et al., 1995; Swanson et al., 1991). The chronological appearance of gonadotropes (FSH/LH cells), GnRH-Rs, GnRH cells, and GnRH fiber projections into the pituitary provides important insights into the understanding of the complete developmental process of the hypothalamus-pituitary relationship in teleosts (Parhar, 1997).

## Feedback Regulations of the HPG Axis by Sex Steroids

The HPG axis forms a closed loop to maintain homeostasis. Alterations in gonadal activity give feedback to the hypothalamus and pituitary through the changes in the circulating plasma levels of sex steroids. The mechanism of the positive and negative actions of sex steroids over the HPG axis, and the GnRH system in particular, has been a long standing question. Despite substantial efforts in the past decades, no concrete evidence of sex steroid receptors in GnRH1 neurons has been shown, although the positive/negative effects of sex steroids on GnRH1 neurons are obvious (Herbison, 2008; Richardson et al., 2004). However, after the findings of kisspeptins and GnIH as new players in the HPG axis, accumulated information suggests that these two hypothalamic neuropeptides mediate the effects of various internal/external environmental signals, including sex steroids, in the HPG axis of vertebrates (Parhar et al., 2012).

Particularly, involvement of kisspeptins and their receptors in the sex steroid feedback mechanism is now evident in fish (Parhar et al., 2012). Ovariectomy diminishes kiss1 gene expression in the Hv neuronal population in the medaka, and E2 replacement rescues it (Kanda et al., 2008; Mitani et al., 2010). Interestingly, in the zebrafish and goldfish, similar positive effects of estrogen are seen in the kiss2 neurons in the ventral hypothalamus and POA populations, respectively (Kanda et al., 2012; Servili et al., 2011). The sex steroid-sensitive kisspeptin neuronal populations express estrogen receptors (ER $\alpha$  in the medaka and ER $\alpha$ ,  $\beta$ 1 and  $\beta$ 2 in the goldfish), and are thus directly regulated by sex steroids.

These data indicate that kisspeptin neurons mediating the positive feedback differ in different species regardless of the kisspeptin form they express. Therefore, information on the anatomy of the kisspeptin systems (Ogawa and Parhar, 2013) and the regulation of each kisspeptin gene (Kitahashi and Parhar, 2013) is important to understand the roles of kisspeptin in a species. Notably, a negative feedback by sex steroids on teleost kisspeptin neurons has not been shown. This is different from the case in mammals, where kisspeptin neurons in the AVPV and Arc mediate the positive and negative feedbacks, respectively (Smith et al., 2006). Therefore, the fish kisspeptin system might receive only positive but not negative feedback by sex steroids (Figure 3).

Although there are no fish studies that report the effects of sex steroids on the GnIH system, mammalian studies have shown possible involvement of the GnIH system in the negative feedback regulation of the HPG axis (Parhar et al., 2012). However the information is still controversial. A subset of GnIH neurons expresses ER $\alpha$  in the Syrian hamster and mice, suggesting a role of E2 in the regulation of GnIH neurons (Kriegsfeld et al., 2006; Molnar et al., 2011). Indeed, treatment with E2 increases c-fos expression in GnIH neurons, indicating the involvement of GnIH in the negative feedback action of sex steroids (Kriegsfeld et al., 2006). Conversely, GnIH mRNA levels in ovariectomized mice are reduced by E2 treatment, suggesting the role of GnIH in positive feedback in mice (Molnar et al., 2011). Furthermore, no effects of E2 treatment on GnIH mRNA levels were observed in ovariectomized rats and ewe (Quennell et al., 2010; Smith, 2008). Therefore, the effects of sex steroids on GnIH neurons might be species-, treatment-, or physiological condition-dependent.

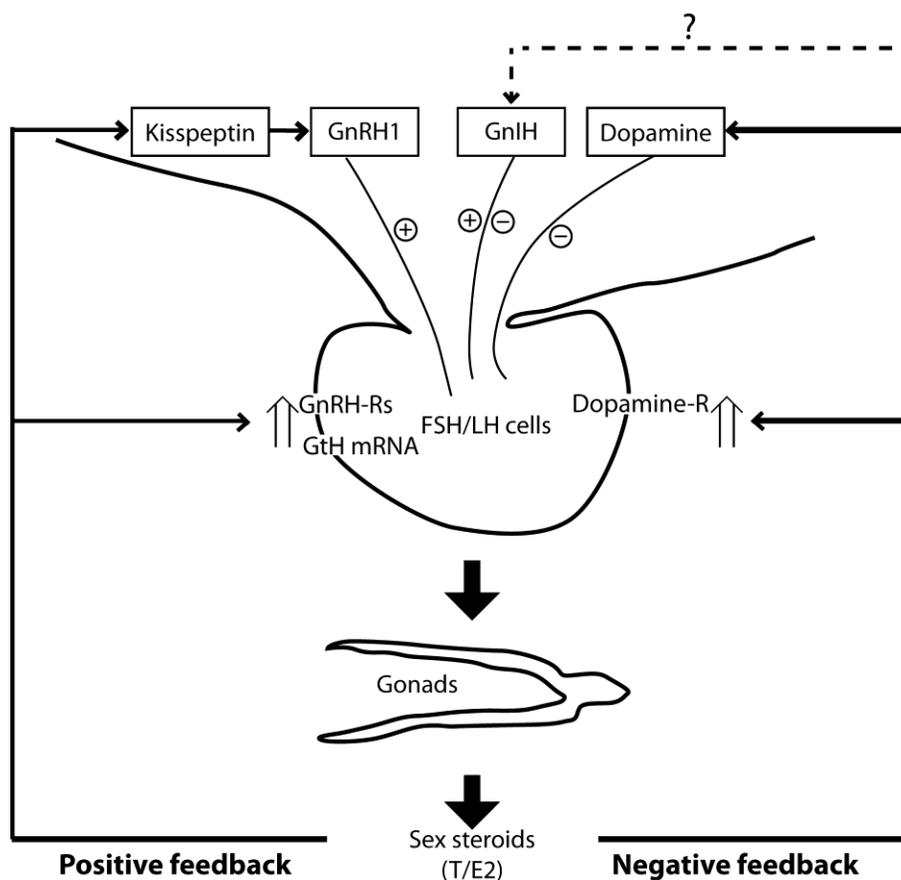


Figure 3. A diagram showing summary of the HPG axis in teleosts. GnRH1 plays a major stimulatory role in GtH secretion, while kisspeptin stimulates GnRH1 release. On the other hand, dopamine plays an important role as an inhibitor of GtH secretion at least in some species. GnIH inhibits GtH release but stimulates GtH synthesis. In terms of sex steroid feedback, the positive feedback seems to be mediated by the stimulatory effects of sex steroids on kisspeptin neurons and the synthesis of GnRH-R in the pituitary. Sex steroids also directly stimulate GtH synthesis at the pituitary level. The negative feedback is mediated by the stimulatory effects of sex steroids on the synthesis of dopamine in the brain and dopamine D2 receptor in the pituitary. Whether GnIH neurons are regulated by sex steroids is unknown. Notably, the strength of each component in the whole system seems to vary depend on species, maturational stage, season, and environmental conditions.

On the other hand, T administrations stimulate the hypophysiotropic population (NPOav) of dopaminergic neurons in the female European eel (Weltzien et al., 2006), suggesting that dopaminergic neurons are involved in the negative feedback regulation of the HPG axis (Figure 3). Further studies in teleosts are required to demonstrate if teleost GnIH neurons also mediate the negative feedback action of sex steroids in the HPG axis.

The pituitary gonadotropes themselves are also the sites of sex steroid feedback. Single-cell real-time PCR combined with laser-capture microdissection showed that individual FSH and LH cells of the male tilapia express multiple types of sex steroid receptors, including  $ER\alpha$ ,  $ER\beta$ ,  $AR\alpha$ , and  $AR\beta$  (Kitahashi et al., 2007). Interestingly, the combinations of sex steroid receptors detected in individual gonadotropes and the expression levels of each receptor are significantly different between sexually immature and mature fish (Kitahashi et

al., 2007). Indeed, the effects of E2 on GtH transcription differ among maturational stages in the masu salmon (Ando et al., 2004), suggesting that changes in the combination of sex steroid receptors decide the response of gonadotropes to circulating sex steroids. Increased levels of GnRH-R and dopamine D2 receptor mRNAs in estradiol-treated pituitary suggest that sex steroids are involved in both positive and negative feedback mechanisms by altering the sensitivity of gonadotropes to GnRH and dopamine (Levavi-Sivan et al., 2006).

## Conclusion

A variety of neuropeptides and neurotransmitters, including GnRHs, kisspeptins, and GnIH are secreted from the hypothalamus to regulate the synthesis and release of GtHs in a cooperative manner. Kisspeptins are involved in the positive feedback of sex steroids, and play an important role in the onset of puberty in fish species, as in mammals. The inhibitory role of GnIH on GtH release seems to not be consistent across teleosts and mammals. In fact, *in vivo* administrations of GnIH rather stimulate GtH synthesis in certain maturational stages at least in some species. However, GnIH has an important role in the control of the HPG axis as a member of the inhibitory factors of gonadotropin release. Dopaminergic inhibition of gonadotropes is also largely species-specific, but seems to work as a mediator of the negative feedback action of sex steroids. Therefore, the inhibition of GtH by GnIH and dopamine varies among teleost species. The variation in GtH regulatory mechanism among species is probably the basis of the large variations in reproductive strategies in teleost species. To understand the hypothalamic regulation of pituitary gonadotropes in teleosts, a comparative aspect of studies in different species is very important. Understanding the interactions among hypothalamic neurons is also one of the primary goals of future studies.

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