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[REVIEW]

Molecular Regulation of Gonadotropin Secretion by Gonadotropin-Releasing Hormone in Salmonid Fishes

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ABSTRACT—Gonadotropin-releasing hormone (GnRH) plays a central role in the control of reproductive function in vertebrates. In salmonids, salmon GnRH (sGnRH) secreted by preoptic GnRH neurons regulates gonadal maturation through stimulation of synthesis and release of pituitary gonadotropins (GTHs). In addition, several lines of our evidence indicate that sGnRH is involved in spawning behavior, and serves to integrate the gonadal maturation with the reproductive behavior. A growing number of studies show that the effects of GnRH are mediated by multiple subtypes of GnRH receptors, successive multiple signaling pathways, and finally multiple transcription factors which act cooperatively to stimulate transcription of GTH subunit genes. This complex regulatory system of the action of GnRH may serve as a molecular basis of divergent physiological strategies of reproductive success in various vertebrate species. In this article, recent data on the molecular mechanisms of action of GnRH are reviewed with special reference to the regulation of synthesis and release of GTHs in the pituitary of salmonids to elucidate the multifunctional action of GnRH.

Key words: GnRH, GnRH receptor, GTH, gene expression, signal transduction pathway, salmon, seasonal reproduction, spawning migration

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a decapeptide that regulates synthesis and release of two gonadotropins (GTHs), follicle-stimulating hormone (FSH) previously referred to as GTH I and luteinizing hormone (LH) previously as GTH II, and thereby serves as a principal neuroendocrine mediator which controls reproductive function in a wide-range of vertebrate species. Since the first isolation of GnRH from pig and sheep hypothalami (Matsuo *et al*., 1971; Amoss *et al*., 1971), the number of GnRH forms rapidly increased, so that at least 14 different molecular forms are now known in vertebrates (Vickers *et al*., 2004). Furthermore, unique GnRH forms were found in procordates (Powell *et al*., 1996; Adams *et al*., 2003) and in octopus (Iwakoshi *et al*., 2002).

It is now evident that there are two or three GnRH forms in a single vertebrate species. One form of GnRH, so-called hypothalamic GnRH, regulates gonadal maturation through stimulation of pituitary gonadotropes. The second form of GnRH is chicken GnRH-II (cGnRH-II), which is highly conserved from fish through mammals. cGnRH-II neurons are localized in the midbrain tegmentum and send their axons widely throughout the central nervous system. cGnRH modulates sexual behavior in some vertebrate species (Millar, 2003). In modern teleosts such as perciformes and pleuronoctiformes, salmon GnRH (sGnRH) is produced as the third form of GnRH in the neuronal groups that are localized rostrally along the terminal nerve. These neurons project their axons throughout the various brain loci, and are shown to have neuromodulatory functions (Oka, 2002; Saito *et al*., 2003).

There are two GnRH forms, sGnRH and cGnRH-II, in

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the brains of salmonid fishes. We have focused on the neuroendocrine regulation of spawning migration in salmonids, in particular the biological significance of sGnRH in the control of homing behavior as well as sexual maturation. A number of studies conducted in salmonids showed that sGnRH has a central role in the regulation of synthesis and release of GTHs, and thus promotes the gonadal maturation (Amano *et al*., 1997).

In addition to the hypophysiotropic action, recent studies of pre-spawning salmons that were captured on the route of homing migration indicated that sGnRH accelerates homing behavior. Administration of GnRH analog (GnRHa) shortened the duration of homing behavior in sockeye salmon (Sato *et al*., 1997; Kitahashi *et al*., 1998b) and chum salmon (Kitahashi *et al*., 2001). It is thus conceivable that sGnRH regulates both homing behavior and gonadal maturation, and synchronizes these functions to accomplish the reproductive success (Onuma *et al*., 2005).

As described above, GnRH is the principal regulator for both the reproductive behavior and sexual maturation in many vertebrate species including salmonids, although molecular mechanisms of the multifunctional action of GnRH remain to be elucidated. Recent reports showed that multiple genes encoding GnRH receptors (GnRH-Rs) are present in a single species (Lethimonier *et al*., 2004; Millar *et al*., 2004). Several GnRH-R subtypes with different structural characteristics are expressed in the brain, pituitary and various peripheral organs. It has become increasingly more obvious that multiple signal transduction pathways are activated inside the target cells by binding of GnRH to its receptors, indicating that the complex GnRH signals in the target cells are responsible for the multiple functions of GnRH in reproduction. These intracellular processes that mediate the action of GnRH are, however, largely unknown at present, particularly in the brain. Since there were abundant studies that focused on the action of GnRH in the pituitary, that is, control of GTH secretion in mammals (Kaiser *et al*., 1997) and teleosts (Ando *et al*., 2001a; Yaron *et al*., 2003), we intend to review recent data on the molecular mechanisms of the action of GnRH with special reference to the regulation of GTH secretion in the pituitary of salmonids.

GnRH AND GTH DURING REPRODUCTIVE CYCLE

Seasonal activation of sGnRH gene expression

Two genes (sGnRH-I and -II) encode sGnRH precursors in salmonids (Higa *et al*., 1997). They are co-expressed in almost all sGnRH neurons in the forebrain (Amano *et al*., 1998). However, the expression level of sGnRH-II gene is much higher than that of sGnRH-I gene (Ando *et al*., 2001b; Kitahashi *et al*., 2004; Onuma *et al*, 2005).

In masu salmon, the amounts of sGnRH mRNAs are high during winter through spring in the prepubertal stage, and decline toward summer, and then increase again in the spawning period (Ando *et al*., 2001b; Kitahashi *et al*., 2004). These changes correspond well with the content of sGnRH in the forebrain (Amano *et al*., 1992, 1993). In contrast, the amount of sGnRH in the pituitary gradually increases with sexual maturation and reaches its maximum in the spawning period. The augmented expression of sGnRH genes in the prepubertal stage suggests that there is a neuromodulatory action of sGnRH that is involved in homing migration, because masu salmon initiate homing migration at this stage.

The expression of gene encoding prolactin (PRL), which is involved in teleostean adaptation to fresh water, was stimulated by GnRHa treatment in masu salmon pituitaries at the prepubertal stage in early spring (Bhandari *et al*., 2003). This temporal stimulation of PRL gene expression by GnRHa may relate to the physiological action of sGnRH involved in the initiation of upstream migration mentioned above.

The coincidental elevation of the amounts of sGnRH and GTH subunit mRNAs in the pre-spawning and spawning periods is certainly important for the final sexual maturation and upstream migration to the spawning ground. In homing chum salmon, expression of sGnRH genes was elevated in almost all forebrain loci during the last phase of spawning migration (Fig. 1) (Onuma *et al*., 2005). These results support the notion that sGnRH neurons are activated during spawning migration, and coordinately regulate migratory

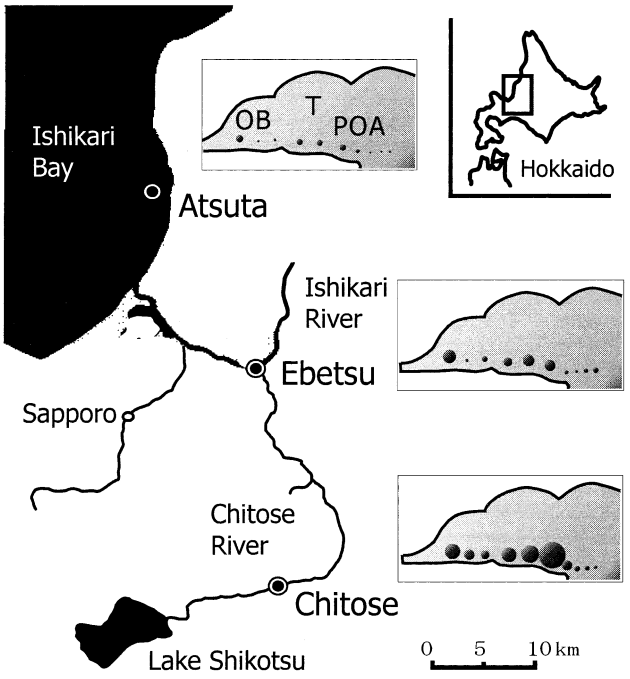

Fig. 1. Activtion of sGnRH gene expression in the brain of female chum salmon during upstream migration. Fish were sampled in three points on the route of upstream migration to the hatchery in 1997: Atsuta, coastral area; Ebetsu, midway of the river; Chitose, the Chitose Salmon Hatchery. The contents of sGnRH-I and -II mRNAs were determined in ten different loci in the brain by real-time PCR assays (see detail in Onuma *et al*., 2005). The data are represented by the circle size. OB, olfactory bulb; T, telencephalon; POA, preoptic area.

behavior and the final sexual maturation. Furthermore, we hypothesized that sGnRH is involved in the onset of the motivated state of homing chum salmon in addition to the promotion of gonadal maturation at the beginning of spawning migration. By use of a quantitative real-time PCR technique, we are now examining whether the expression of sGnRH genes is activated in the brain of chum salmon when they leave the Bering Sea for their natal river.

Differential secretion of FSH and LH

FSH and LH are produced in independent adenohypophysial cells in salmonids (Nozaki *et al*., 1990a; Naito *et al*., 1991), and are differentially released during the reproductive cycle. Many studies on pituitary contents (Suzuki *et al*., 1988; Sumpter and Scott, 1989; Nozaki *et al*., 1990a, b; Naito *et al*., 1991) and plasma levels (Suzuki *et al*., 1988; Swanson *et al*., 1989; Sumpter and Scott, 1989; Oppen-Berntsen *et al*., 1994; Slater *et al*., 1994; Prat *et al*., 1996; Saligaut *et al*., 1998; Gomez *et al*., 1999) of GTHs showed that the FSH levels are elevated during spermatogenesis and vitellogenesis, whereas the LH levels increase during the final sexual maturation.

In masu salmon, we recently examined GTH release activity of primary pituitary cell cultures at four reproductive stages in March (initiation of sexual maturation), May (early maturation), July (pre-spawning), and September (spawning period) (Ando *et al*., 2004). FSH levels in the culture medium increased with sexual maturation and peaked in September, whereas LH release remained low until July and considerably increased in September. These results indicate that the differential regulation of releases of two GTHs can be achieved in part *in vitro* condition without hypophysiotropic stimuli and neuronal inputs such as dopaminergic and γ-aminobutyric acid (GABA)-ergic (Yaron *et al*., 2003). It thus appears that two different types of gonadotropes (FSH and LH cells) release GTH autonomously in different manners.

Recent studies on the temporal expression of GTH subunit genes during sexual maturation showed somewhat different patterns from that of release activity. The amounts of FSHβ and LHβ mRNAs increase with sexual maturation, and reach considerably high values at spawning. Nevertheless, the increase in the content of FSHβ mRNA is initiated at early stage of gametogenensis and the elevation of that of LHβ mRNA is initiated later during gametogenensis (Gomez *et al*., 1999; Kitahashi *et al*., 2004). In the primary pituitary cells of masu salmon, the amounts of α2, FSHβ, and LHβ mRNAs increased from March through May and reached their maximum at the pre-spawning stage in July (Ando *et al*., 2004). The amounts of α2 and FSHβ mRNAs then declined in September, while that of LHβ mRNA remained at high levels.

The changes in the GTH synthesis and release activities apparently showed differential GTH secretion: FSH production is initiated at the early stage of gametogenesis and augmented in parallel with sexual maturation, and FSH release follows this change. In contrast, LH production is initiated at the early stage of gonadal maturation, but most of newly synthesized LH is accumulated in the adenohypophysis during the pre-spawning period for the massive release at the spawning.

There were many previous studies that focused on the mechanisms controlling the differential secretion of FSH and LH. It is well established in mammals that GTH synthesis and release are regulated by multiple factors including GnRH, sex steroids and gonadal peptides such as activin and inhibin. In fish, however, much less is known about this process. The fish, particularly seasonal spawner, provide an interesting model for investigation of the mechanism of differential GTH secretion, because their sexual maturation progress slowly in concert with environmental cues, such as day length and water temperature. In most salmonid species, gonadal maturation is initiated in spring and accomplished in autumn, and is accelerated by exposure to short photoperiod (Amano *et al*., 1995). A use of fish at various reproductive stages allows us to examine different regulatory mechanisms that depend on a certain reproductive stage. The salmonid fishes thus provide a good model for investigation of the regulatory mechanisms of differential synthesis and release of the two GTHs.

Effects of GnRH on GTH secretion

The role of GnRH as an inducer of LH release was established in fish (Trudeau, 1997; Yaron *et al*., 2003), whereas less attention was paid to the regulation of FSH release. In salmonids, GnRHa selectively stimulated FSH release from the pituitary of immature rainbow trout, but it stimulated LH release in mature fish (Kawauchi *et al*., 1989; Breton *et al*., 1998). sGnRH-induced releases of FSH and LH from primary pituitary cells were reported in maturing coho salmon (Dickey and Swanson, 2000) and in pre-spawning masu salmon (Ando *et al*., 2004). In the primary pituitary cells from spawning masu salmon, sGnRH stimulated release of LH but not FSH (Ando *et al*., 2004). It therefore appears that sGnRH stimulates releases of FSH and LH at different maturational stages (Table 1).

The stimulatory role of GnRH in GTH subunit gene expression was shown in various fish species including salmonids (Khakoo *et al*., 1994; Xiong *et al*., 1994a; Melamed *et al*., 1996, 2002; Ando *et al*., 1999; Hassin *et al*., 1998, 2000; Gur *et al*., 2001; Rosenfeld *et al*., 2001; Sohn *et al*., 2001; Kandel-Kfir *et al*., 2002; Klausen *et al*., 2002a; Chong *et al*., 2004). In salmonids, GnRHa that was implanted into the dorsal muscle increased the amount of α2 and LHβ mRNAs, but not FSHβ mRNA in the pre-spawning sockeye salmon (Kitahashi *et al*., 1998a) and masu salmon (Kitahashi *et al*., 2004). However, in the primary pituitary cells prepared from the pre-spawning female masu salmon, sGnRH increased the amount of α2 and FSHβ mRNAs but not LHβ mRNA (Ando *et al*., 2004). Furthermore, sGnRH increased only the levels of FSHβ mRNA in the primary pituitary cells of maturing coho salmon (Dickey and Swanson, 2000). These results indicate that the action

Table 1. Effects of GnRH and E2 on release and synthesis of GTH in salmonids at three different reproductive stages*.

	Release			Synthesis		
FSH	GnRH	GnRH & E2	E2	GnRH	GnRH & E2	E2
Maturing	+	++	–	+	–	–
Pre-spawning	+	++	–	+	–	–
Spawning	–	-	–	–	–	–
LH	GnRH	GnRH & E2	E2	GnRH	GnRH & E2	E2
Maturing	+	+	–	–	+++	++
Pre-spawning	+	++	+	–	++	++
Spawning	+++	+++	+		–	–

+++ strong stimulation, ++ moderate stimulation, + weak stimulation, – no effect

* It should be noted that the relative intensities of effects are reliable only within the same hormone and function.

of GnRH on expression of GTH subunit gene depends on the subunit gene and reproductive stage (Table 1).

The effects of GnRH on GTH gene expression were different between *in vivo* and *in vitro* conditions, indicating that there are additional endogenous factors that modulate the action of GnRH on GTH gene expression. Considering that the responsiveness to GnRH of gonadotropes is highly dependent on the reproductive stage, gonadal sex steroid hormones are strong candidates for the modulation of the action of GnRH.

Modulation of the action of GnRH by E2

Sex steroid hormones have both positive and negative effects on GTH secretion, depending on the mode of administration and the reproductive stage of animal. In immature and maturing salmonids, aromatizable androgens and estradiol-17β (E2) stimulate LH synthesis (Crim and Evans, 1979; Crim *et al*., 1981; Trinh *et al*., 1986; Xiong *et al*., 1994b; Borg *et al*., 1998; Dickey and Swanson, 1998). In contrast, negative or no effects on LH synthesis of sex steroids were reported in mature fish. A gonadoectomy of spermiating coho salmon resulted in an increase in the plasma LH levels, suggesting a negative feedback control of LH secretion by sex steroids (Larsen and Swanson, 1997). The treatment of pituitary cells from masu salmon with E2 and testosterone (T) significantly increased the amount of LHβ mRNA in the maturing and the pre-spawning stages, but significant changes were not observed in the spawning period (Ando *et al*., 2004). In the same fish samples, E2 showed a weak stimulatory activity of LH release in the pre-spawning and the spawning stages (Table 1).

Beside many studies on the regulation of LH secretion by GnRH or sex steroids, a few studies investigated effects of a co-treatment with GnRH and sex steroid to clarify functional interaction between these factors. Pre- or co-treatment with E2 or T potentiated the action of GnRH to induce LH release in rainbow trout (Crim and Evans, 1983; Weil and Marcuzzi, 1990). Similarly, sGnRH-induced LH release was synergistically stimulated by a co-treatment with E2 in the primary pituitary cells of masu salmon in all stages during sexual maturation (Ando *et al*., 2004). The amount of LHβ mRNA was also extensively elevated by the co-treatment with E2 in the maturing stage, although sGnRH alone did not have any effects on the amount of LHβ mRNA. Therefore, the synergistic stimulation by the combination of GnRH and E2 seems to be evident in the reproductive stage-specific LH synthesis and release in the intact pituitary (Table 1), in addition to the stimulatory role of GnRH in LH release and of E2 in LH synthesis.

The effects of sex steroids on secretion of FSH received much less attention. It seems to be more complex. In maturing coho salmon, in vivo treatment with E2 or T had little effects on the pituitary contents of FSH and FSHβ mRNA, whereas the plasma FSH level was markedly decreased by the treatment, suggesting a negative feedback by sex steroids on FSH synthesis at the translational level (Dickey and Swanson, 1998). Gonadoectomy of pre-spawning and spermiating coho salmon resulted in increases in the plasma FSH levels, supporting the negative feedback control on FSH mentioned just above (Larsen and Swanson, 1997). However, in Atlantic salmon, the pituitary and plasma levels of FSH in castrated fish at the spawning stage were lower than those in sham-operated fish, and a treatment with T increased the FSH levels, indicating a positive feedback effect of sex steroid on FSH secretion (Borg *et al*., 1998). E2 and T did not have any effects on the amount of FSHβ mRNA, regardless of coexistence of sGnRH, in the primary pituitary cells of masu salmon (Ando *et al*., 2004). These results suggest that other factors are involved in stimulation of FSH synthesis in the pituitary (Table 1). Nevertheless, sGnRH stimulated FSH release in synergism with E2 in the maturing and pre-spawning stages, indicating that sGnRH and E2 participate in part in the release of FSH during sexual maturation (Table 1).

GnRH RECEPTORS

The action of GnRH is mediated through binding of

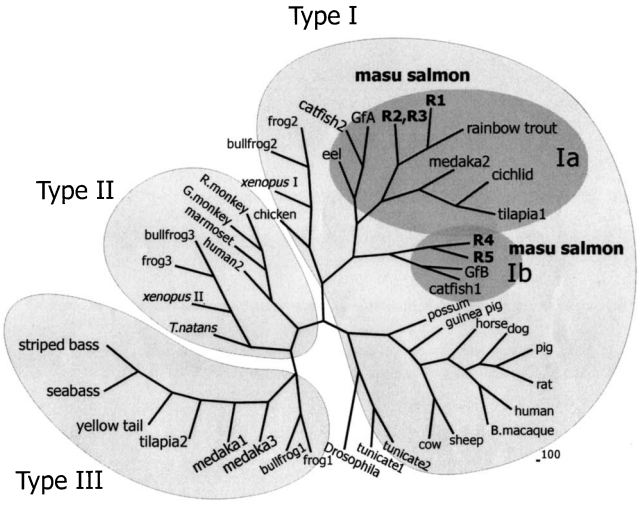

Fig. 2. Phylogenetic analysis of GnRH-R in vertebrates. A phylogenetic tree was generated by the neighbor-joining method using the partial amino acid sequences of GnRH-Rs, spanning from the extracellular N terminal domain to the third transmembrane domain. The *Drosophila* GnRH-R homolog was used as an outgroup.

GnRH to a single class of G protein-coupled membrane receptors in the target cells. Since the first molecular characterization of mouse GnRH-R (Tsutsumi *et al*., 1992), cDNAs encoding GnRH-R were cloned in various vertebrate species. A phylogenetic analysis of these GnRH-R sequences indicates the presence of three main types, which were termed as type I, type II, and type III receptors (Fig. 2) (Millar *et al*., 2004). Among the three types, fish GnRH-R belongs to the type I and type III. It is now evident that multiple GnRH-R types present in single species. In mammals, type I and type II GnRH-Rs have distinct ligand selectivity. The type I receptor is highly sensitive to mammalian GnRH, whereas the type II receptor shows a clear preference for cGnRH-II. In non-mammalian vertebrates, multiple GnRH-Rs in a single species, however, do not have such clear different selectivity for native GnRH ligands. All types of GnRH-Rs in teleosts have a particular preference to cGnRH-II, followed by sGnRH and then the third endogenous GnRH when identified (Lethimonier *et al*., 2004). Therefore, ligand-receptor relationships of the multiple GnRH-Rs remain unclear in teleosts. Information on distribution and expression levels of GnRH-Rs in target organs is required to determine if the multiple GnRH-R types have different functions in terms of the action of GnRH.

In salmonids, only type I GnRH-R was first identified in the brain of rainbow trout (rtGnRH-R) (Madigou *et al*., 2000). Thereafter, the second mRNA isoform, which is generated by alternative promoter usage and splicing, was characterized (Madigou *et al*., 2002). We recently demonstrated in masu salmon that five different GnRH-R genes, termed as msGnRH-R1, R2, R3, R4, and R5, are expressed in the brain, the pituitary and other peripheral tissues with different patterns (Jodo *et al*., 2003). A splicing variant of msGnRH-R1 (R1-v) is also expressed in these tissues. All these receptors are type I receptors, but are divided into two subtypes, one subtype (type Ia) includes R1, R2, and R3 and the other (type Ib) includes R4 and R5 (Fig. 2). The identity of nucleotide sequences among R1, R2 and R3 is 96–99%, while that of R4 and R5 is 81%. The identity between the two subtypes decreases to 59–71%.

Ia subtype includes the goldfish GnRH-R (GfA, Illing *et al*., 1999) and catfish GnRH-R (cfGnRH-R2, Bogerd *et al*., 2002), while Ib includes GfB and cfGnRH-R1. Because salmonids, goldfish and catfish are rather ancient tetraploid teleosts, the two subtypes may arise from a genomic duplication. Recently, another type of GnRH-R that belongs to type III receptor was identified in rainbow trout (Lethimonier *et al*., 2004). It is most probable that there is also a type III GnRH-R in masu salmon. If so, there are at least six different subtypes of GnRH-R in masu salmon. It is of considerable interest and importance to determine how these multiple GnRH-R subtypes mediate GnRH signals to different action during reproductive cycle as discussed above, and whether they have different roles in terms of the action of GnRH.

The different effects of GnRH on synthesis and release of two GTHs may be attributed in part to changes in expression of GnRH-Rs in FSH and LH cells. We therefore examined by real-time PCR seasonal variations in expression of the five msGnRH-R genes in the pituitary of masu salmon during the reproductive cycle. All five subtypes of msGnRH-R genes were expressed in the pituitary, although R4 mRNA was dominant. Interestingly, the expression patterns of five msGnRH-R genes differed among different subtypes. Among them, R4 mRNA increased only in the pre-spawning period, when expression of LH subunit genes was stimulated by GnRHa, suggesting that the R4 subtype is involved in the GnRH-induced LH synthesis (Jodo *et al*., in preparation). Other subtype mRNAs increased in different periods, so that they can be involved in other action of GnRH in the pituitary, such as GnRH-induced PRL gene expression as described previously. To obtain more accurate information on the function of the multiple GnRH-R subtypes, cell type specific distribution of the GnRH-Rs in the pituitary is currently under investigation.

SIGNAL TRANSDUCTION PATHWAY

GnRH activates multiple signal transduction pathways such as Ca^{2+} and cAMP signaling through binding to GnRH-R, which is able to couple to multiple G proteins ($G_{q/11}$, G_S and $G_{i/o}$) (Stanislaus *et al*., 1998; Ruf *et al*., 2003; Millar *et al*., 2004). In general, regulation of GTH secretion by GnRH is primarily mediated by Ca^{2+} signaling via $G_{q/11}$ (Grosse *et al*., 2000). Activation of $G_{q/11}$ proteins stimulates phospholipase C to generate inositol trisphosphate and diacylglycerol. Increases of these signaling messengers lead to activation of protein kinase C (PKC) and also an increase in intracellular Ca^{2+} concentration. These two secondary signal mediators are involved in GnRH-induced GTH release and syn-

thesis (Klausen *et al*., 2002b). Afterwards, mitogen-activated protein kinase (MAPK) that locates downstream of PKC plays a role in the regulation of GTH subunit gene expression in response to GnRH. PKC/MAPK and Ca^{2+} influx differentially control expression of three GTH subunit genes (Ando *et al*., 2001a). Recent studies indicated that Ca/calmodulin-dependent kinase II (Ca/CaMKII) pathway is also involved in the GnRH-induced GTH subunit gene expression (Haisenleder *et al*., 2003).

In fish, GnRH is also involved in the regulation of GTH release and synthesis through multiple signaling pathways including PKC, Ca^{2+}, and PKA (Ando *et al*., 2001a; Klausen *et al*., 2002b; Yaron *et al*., 2003). Most of these studies were conducted in goldfish and tilapia, and much less are known in salmonids. We examined whether the PKC/MAPK and Ca^{2+} influx signaling are involved in the GnRH-stimulated LHβ gene expression using a gonadotrope-derived cell line, αT3-1 (Ando *et al*., 1999, 2001a).

GnRHa specifically stimulated the activity of promoter of chinook salmon LHβ gene when transfected in αT3-1 cells. A specific L-channel agonist, Bay K8644 did not stimulate LHβ promoter activity, and an L-channel antagonist, nimodipine, did not block the GnRH-stimulated LHβ gene expression. Furthermore, a MAPK inhibitor, PD098059, almost eliminated this stimulation. These results suggest that PKC/MAPK pathway but not Ca^{2+} influx mediates the stimulation of LHβ gene expression by GnRH. However, it is possible that signal transduction pathways from GnRH-R to LHβ gene are not identical to that in intact gonadotropes in a salmon pituitary. Further studies are necessary to define the significance of PKC/MAPK pathway in the GnRH-induced LHβ gene expression in salmonids. Nevertheless, the transfection experiment using the heterologous cell system provided important information on molecular mechanism of the action of GnRH: GnRH can stimulate salmon LHβ gene transcription by activation of DNA-binding transcription factors through PKC/MAPK pathway.

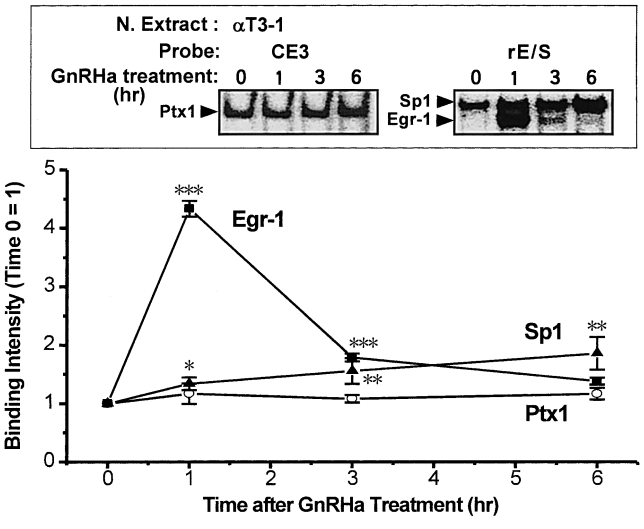

Fig. 3. Transient increases in the binding activities of Egr1 and Sp1 in response to GnRHa in αT3-1 cells. Nuclear extracts of αT3-1 cells were prepared at different time after the treatment with 100nM GnRHa (des-Gly^{10}, [D-Ala^{6}]-GnRH ethylamide). The binding activities of Egr1, Ptx1, and Sp1 were examined by gel mobility shift assays using CE3 (a Ptx1 binding element in the rat preopiomelanocortin gene promoter, Lamonerie *et al*., 1996) and rE/S (A binding site for Egr1 and Sp1 in the rat LHβ gene promoter, –55 to –35) as probes. Asterisks denote significant difference with respect to the binding intensity at time 0 (* P<0.05, ** P<0.01, *** P<0.001).

TRANSCRIPTIONAL REGULATION OF GTH GENES

Transcription factors involved in the action of GnRH

Extensive studies in mammals showed that transcription factors which are involved in the stimulation by GnRH of GTH subunit genes are different among three GTH subunit genes (Ando *et al*., 2001a; Ruf *et al*., 2003). These include a LIM-homeodomain protein, an Ets-related tran-

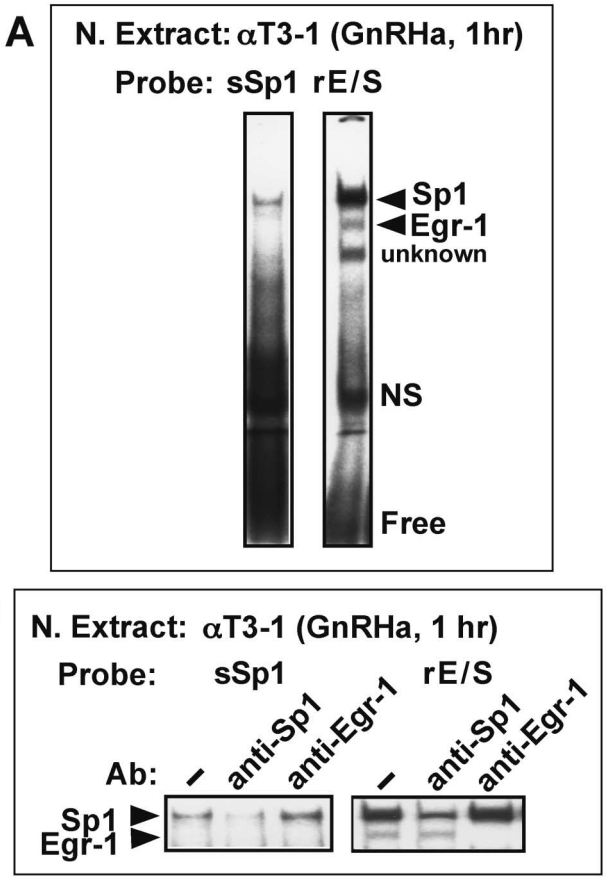

Fig. 4. Binding of Sp1 to a GC-rich sequence (sSp1) in the proximal region of the chinook salmon LHβ gene. (A) Gel shift analysis of the GC-rich element in the salmon LHβ gene (–55 to –35). Nuclear extracts of αT3-1 cells were prepared at 1 hr after the treatment with 100nM GnRHa (des-Gly^{10}, [D-Ala^{6}]-GnRH ethylamide). Gel shift analysis was performed using the sSp1 and the rE/S (see Fig. 3). A band corresponding to Sp1 but not Egr1 was shifted using the sSp1 as a probe. (B) Supershift experiment of the sSp1. The nuclear extract was pre-incubated with antiserum against Sp1 or Egr1. The intensity of the shifted band of sSp1 was decreased by anti-Sp1 antiserum.

scription factor and cAMP response element binding protein (CREB) for α gene, Sp1, early growth response protein 1 (Egr1) and pituitary homeobox 1 (Ptx1) for LHβ gene, and activating protein-1 (AP-1) and nuclear factor-Y (NF-Y) for FSHβ gene. GnRH thus regulates GTH subunit genes by activating different sets of transcription factors through multiple signal transduction cascades.

Several additional transcription factors act in concert to mediate GnRH signals to LHβ gene, with Egr1 serving an essential function. Egr1 stimulates LHβ gene transcription in response to GnRH in the synergism with Ptx1 and a nuclear receptor, steroidogenic factor-1 (SF-1), which is a mammalian homolog of *Drosophila fushi tarazu* factor 1α (dFTZ-F1α). When GnRH signals come into the nucleus, expression of Egr1 gene, which is an immediately early gene, is rapidly increased (Fig. 3), and Egr1 synergizes with Ptx1 and SF-1 to stimulate LHβ gene. *Cis*-acting DNA elements corresponding to these factors are conserved in mammalian LHβ genes.

In contrast to mammals, there is a paucity of information concerning transcription factors involved in the regulation of fish GTH subunit genes by GnRH (Ando *et al*., 2001a; Yaron *et al*., 2003). Although genomic sequences of fish GTH subunit genes have been determined from several fish species including chinook salmon (α gene, Suzuki *et al*., 1995; LHβ gene, Xiong and Hew, 1991; FSHβ gene, Chong *et al*., 2004), masu salmon (α gene, Gen *et al*., 1997), carp (α gene, Huang *et al*., 1992; LHβ gene, Chang *et al*., 1992), goldfish (LHβ gene, Sohn *et al*., 1999; FSHβ gene, Sohn *et al*., 1998), tilapia (LHβ and FSHβ genes, Rosenfeld *et al*., 1997, 2001), and African catfish (FSHβ gene, Vischer *et al*., 2003), *cis*-acting elements and transcription factors required for GnRH induction were determined only in the chinook salmon LHβ (Ando *et al*., 1999, 2001; Melamed *et al*., 2002) and the tilapia FSHβ genes (Rosenfeld *et al*., 2001; Yaron *et al*., 2003).

Using murine cell lines such as αT3-1 and LβT2 as models, GnRH responsive regions were determined in the LHβ gene promoter of chinook salmon. A proximal region (–258 to –199) that contains a binding site for Ptx1 was important in the GnRH stimulation (Ando *et al*., 1999). Furthermore, other distal Ptx1 sites mediated GnRH-induced LHβ gene transcription (Melamed *et al*., 2002). It should be noted that there is no functional binding sites for Egr1 in the promoter of LHβ gene, and Egr1 is ineffective to stimulate transcription of LHβ gene (Le Drean *et al*., 1997).

Interestingly, in the proximal region of salmon LHβ promoter, there are two GC rich sequences (–41 to –33 and –85 to –77) that are similar to a common consensus binding sites of Egr1 and Sp1. Gel retardation analysis using the proximal GC element as a probe showed that Sp1 but not Egr1 bound to this element (Fig. 4). Site-directed mutagenesis of the two GC rich Sp1 binding sites and also two proximal Ptx1 binding sites (–133 to –127 and –258 to –199) decreased the induction by GnRH, indicating that these Sp1 and Ptx1 sites are involved in stimulation of LHβ gene by GnRH. Since Sp1 binding activity increased after GnRHa treatment in αT3-1 cells (Fig. 3), Sp1 is capable to serve as a direct molecular target of GnRH signals like Egr1. It is highly conceivable that the stimulation of LHβ gene by GnRH is primary mediated by Sp1, which act in synergism with Ptx1 and other transcription factors, such as SF-1 and estrogen receptor (ER) as discussed below.

Regulation of LHβ gene transcription

GnRH synergizes with E2 to stimulate LH synthesis in the pituitary of salmonids. The effects of E2 are mainly mediated by ER, a transcription factor which regulates target genes through binding to estrogen responsive elements (EREs) in their promoters. The presence of two functional EREs, pERE (–274 to –261) and dERE (–2736 to –2655), were reported in the chinook salmon LHβ promoter (Liu *et al*., 1995; Xiong *et al*., 1994b). They cooperatively stimulate LHβ gene transcription. In addition, SF-1 synergized with the

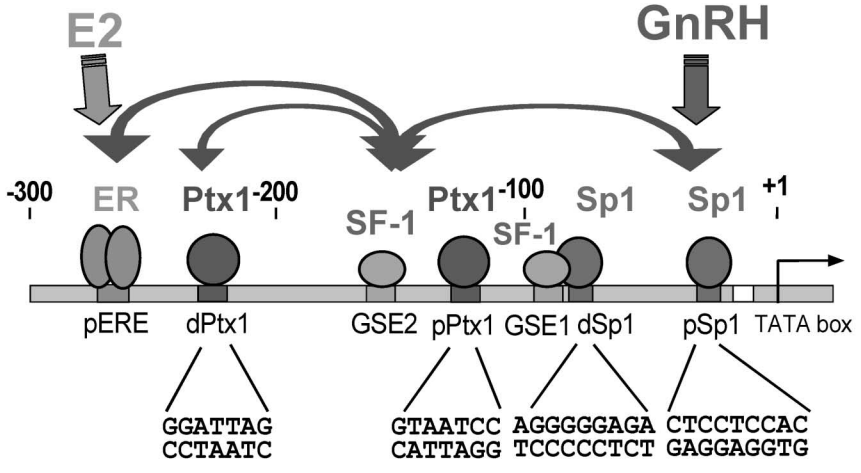

Fig. 5. Proposed model of molecular mechanisms of cooperative regulation of salmon LHβ gene by GnRH and E2. GnRH signals activate Sp1 to interact with Ptx1 and SF-1. Furthermore, E2 stimulates LHβ gene transcription through pERE. ER synergizes with SF-1, facilitating the functional interaction between the GnRH and E2 signals to enhance LHβ gene transcription.

ER through binding to a proximal SF-1 binding site (gonadotrope-specific element, GSE2, Fig. 5), and then stimulated LHβ gene transcription dramatically (Le Drean *et al*., 1996). Since SF-1 was reported to interact with Sp1 (Kaiser *et al*., 2000; Sugawara *et al*., 2000), protein-protein interactions among ER, SF-1, Sp1, and Ptx1 is most probably important for the functional synergism between GnRH and E2 in the stimulation of LHβ gene expression (Fig. 5). The expression of two salmon FTZ-F1 homolog genes, sFF1-I and sFF1-II, was augmented in the pituitaries of chum salmon and sockeye salmon at the late stage of sexual maturation (Higa *et al*., 2000). These results support the physiological significance of SF-1 in the regulation of LHβ gene expression.

Regulation of FSHβ gene transcription

Much less is known about GnRH regulation of FSHβ gene expression. In the ovine FSHβ gene, GnRH activated transcription through two binding sites for activating protein-1 (AP-1). These binding sites are well conserved in mammalian FSHβ gene promoters (Strahl *et al*., 1997; 1998). Furthermore, a novel AP-1 site, which binds AP-1 and NF-Y, was important for the induction of the mouse FSHβ gene by GnRH (Coss *et al*., 2004).

In tilapia, FSHβ promoter activity was stimulated by GnRH when transfected to the primary pituitary cells (Rosenfeld *et al*., 2001), and a GnRH responsive region (–1211 to –821) that contains an AP-1 binding site was determined (Yaron *et al*., 2003). In chinook salmon, the 5' upstream region of FSHβ gene was recently isolated and was shown to be induced by GnRH when transfected into LβT2 cells (Chong *et al*., 2004). *Cis*-acting elements that mediate the response to GnRH remain to be determined.

CONCLUSIONS AND PERSPECTIVES

In the present article, we focused on the molecular mechanisms of the action of GnRH, in particular regulation of GTH subunit gene expression. The data presented in this review indicate that the action of GnRH is mediated by multiple subtypes of GnRH-Rs, successive multiple signaling pathways, and finally multiple transcription factors that act cooperatively to stimulate transcription of GTH subunit genes. During sexual maturation, GnRH synergize with sex steroid hormones to regulate synthesis and release of GTHs, thus increasing the complexity of mediation of GnRH signals. As a result of this complex molecular function stimulated by the action of GnRH, FSH and LH are differentially secreted by pituitary gonadotropes in concert with developmental and environmental stimuli to accomplish the reproductive success. However, it should be noted that the data concerning the GnRH signals presented in our article is only "the tip of the iceberg" of more diverged molecular mechanisms of the action of GnRH. Indeed, we do not know how GnRH regulates α subunit gene in addition to FSHβ gene. The mechanisms of differential regulation of synthesis and release of GTH by GnRH are almost not known.

GnRH in cooperation with E2 stimulates only synthesis of LH in the early stage of sexual maturation, but it plays as a major secretagogue of LH in the spawning period. The effects on synthesis and release by GnRH are probably mediated in part through a common signal transduction pathway where Ca^{2+} serves as a central mediator. Nevertheless, the successive two distinct pathways, one leading to secretory granules and the other leading to nucleus remain unclear. The two pathways may interact with each other, and are most probably balanced to achieve the reproductive stage-specific LH secretion.

There is little information on the molecular mechanisms of the action of GnRH in the brain. GnRH has neuromodulatory roles involved in the regulation of reproductive behavior. Several lines of evidence in our research indicate that this is true in the upstream migration of salmonids. GnRH may regulate neuronal excitability and release of neurotransmitters in target neurons involved in reproductive behavior. However, the target sites and also its molecular action have not yet been determined. These actions may be mediated in part by GnRH-R through a common pathway with that in the gonadotropes. In our current research about expression of the five msGnRH-R genes in the brain, their expression patterns and also regulation was different between the brain and the pituitary. It is thus important to determine accurate distribution of target sites and molecular events in response to GnRH.

Our study in salmonids and other studies in mammals suggest that some transcription factors, such as Ptx1, Sp1, and SF-1 are commonly utilized by LHβ genes in both salmon and mammals, indicating that molecular mechanisms of stimulation of LHβ gene by GnRH are partially conserved across vertebrate evolution. Nevertheless, in the pituitary of salmon, GnRH alone dose not stimulate LHβ gene, whereas it does in mammals. Furthermore, E2 has a negative effect on GTH subunit gene expression in most mammalian cases, whereas it has a stimulatory effect in the fish pituitary. Despite a use of similar sets of transcription factors which play a central role in GTH genes in response to GnRH, their functional interaction is shown to be different between fishes and mammals. These different regulatory mechanisms of the action of GnRH may serve as a molecular basis of divergent physiological strategies of reproductive success in various vertebrate species. Thus, it is of considerable interest and importance to determine a regulatory network of transcription factors regulating GTH gene expression by GnRH in various species of vertebrates, particularly seasonal breeders like salmonids.

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