Hypothalamic – pituitary – gonadal endocrine system in the hagfish

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The hypothalamic – pituitary system, which is specific to vertebrates, is considered to be an evolutionary innovation and seminal event that emerged prior to or during the differentiation of the ancestral jawless vertebrates (agnathans) (Sower et al., 2009). Such an evolutionary innovation is one of the key elements, leading to physiological divergence, including reproduction, growth, metabolism, stress, and osmoregulation.
in subsequent evolution of jawed vertebrates (gnathostomes). The control and the integration of reproductive processes are generally regarded as probably the oldest and original functions of the vertebrate hypothalamic – pituitary system. Of all vertebrate species, both extant and extinct, hagfishes are considered to be the earliest divergent (Forey and Janvier, 1993, 1994), and possess the most primitive hypothalamic – pituitary system. Accordingly, hagfishes are of particular importance in understanding the evolution of the hypothalamic – pituitary system related to vertebrate reproduction. However, until the recent identification of a functional gonadotropin (GTH) in the hagfish and its effects on the gonads (Uchida et al., 2010), it had not been established on whether the hagfish pituitary gland contained tropic hormones of any kind and whether the pituitary gland regulated gonadal functions in the hagfish. Previous studies showed a correlation of the hypothalamic gonadotropin-releasing hormone (GnRH) associated with reproductive cycle in hagfish (Kavanaugh et al., 2005). Additional recent studies on the hagfish steroidogenic hormones and enzymes have revealed that the gonadal functions are under the control of the pituitary gonadotropic activities. Even with these recent data, the hypothalamic – pituitary – gonadal system of the hagfish is still the least understood of all the vertebrates. This chapter summarizes the recent findings on the hypothalamic – pituitary – gonadal endocrine system involved in the reproduction in the hagfish.

Hagfish hypothalamic – pituitary system

Background

The pituitary gland is present in all vertebrates from agnathans (jawless fishes) to mammals and consists of the same two principal elements, the neurohypophysis and adenohypophysis. The neurohypophysis develops from the floor of the diencephalon as an infundibular extension, whereas the adenohypophysis develops from the epithelium that comes in contact with this infundibulum. The enigma of the pituitary gland is that evolution of a composite organ with such a complex double developmental origin must have been associated with some functionally adaptive value and probably resulted due to whole genome duplications that occur early in vertebrate evolution (Smith et al., 2013). Yet, the demonstration of this adaptive value in one of the agnathans, hagfish, has remained elusive. Extensive molecular, biochemical and physiological studies have shown that lamprey, the other member of extant agnathans, have the conserved hypothalamic – pituitary – gonadal axis with some distinct differences (Sower et al., 2009). The pituitary gland along with the three major pituitary hormone families, glycoprotein hormone family that includes the GTHs and thyroid-stimulating hormone (TSH), growth hormone (GH)
family that includes GH and prolactin and the hormones derived from pro-opiomelanocortin such as adrenocorticotropin (ACTH) and melanophore-stimulating hormone (MSH), and their receptors emerged during the early evolution of the ancestral vertebrates (Kawauchi and Sower, 2006; Decatur et al., 2013). In protochordates, neither Ciona nor amphioxus has these pituitary hormone genes in their genomes (Holland et al., 2008).

The hagfish possesses the most primitive hypothalamic–pituitary system. The neurohypophysis of the hagfish is a flattened sac-like structure, whereas the adenohypophysis consists of a mass of clusters of cells embedded in connective tissue below the neurohypophysis (Holmes and Ball, 1974; Hardisty, 1979) (Figures 9.1a and b). The adenohypophysis and the neurohypophysis are completely separated by a layer of connective tissue, and there is no or little anatomical relationship between them (Gorbman et al., 1963; Kobayashi and Uemura, 1972) (Figures 9.1a,b). In addition, there is no clear cytological differentiation between the pars distalis and the pars intermedia (Holmes and Ball, 1974; Hardisty, 1979).

Figure 9.1 (a) Diagrammatically sagittal section of the hagfish pituitary gland. Dark area of the neurohypophysis (NH) shows posterior part of the dorsal wall, where ir-GnRH nerve fibers and AVT nerve fibers are densely accumulated. (b) Nearly mid-sagittal section of the pituitary gland of the brown hagfish, stained with hematoxylin and eosin. (c and d) GTHβ-like immunoreaction in the adenohypophysis of the juvenile (c) and sexually mature (d) brown hagfish stained with anti-hagfish GTHβ. Note that GTH-positive cells are almost absent in c, whereas they are abundant in d. (e) Diagrammatically sagittal section of the hagfish pituitary gland showing the topographic distribution of adenohypophysial cells. Closed circle, GTH cell; open circle, ACTH cell; open triangle, undifferentiated cell and possible GH cell. AH, adenohypophysis; CT, connective tissue; IIIIV, third ventricle. Scale bars: 100 μm. (From Nozaki M. 2013. Front. Endocrinol. 4:200. doi:10.3389/fendo.2013.00200.)
(Figure 9.1b). The question arises whether the simplicity of the hagfish pituitary gland is a primitive or a degenerate feature. For example, some authors have claimed that the pars intermedia seem to have been lost via a secondary degenerative process (Hardisty, 1979; Gorbman et al., 1983). Moreover, until recent identification of a functional GTH in the hagfish pituitary (Uchida et al., 2010), it had not been established whether the hagfish pituitary gland contained adenohypophysial hormones of any kind (Matty et al., 1976; Gorbman, 1983). Because of the simplicity and primitiveness of the pituitary morphology and equivocal data on the adenohypophysial hormones in the hagfish, many researchers had questioned whether there were any functional adenohypophysial hormones (Matty et al., 1976; Gorbman, 1983). On the other hand, arginine vasotocin (AVT), as a single neurohypophysial hormone, was identified in the hagfish (Suzuki et al., 1995). In addition, the presence of GnRH has been suggested in the hagfish hypothalamus by both radioimmunoassay and immunohistochemistry (Braun et al., 1995; Sower et al., 1995; Oshima et al., 2001; Kavanaugh et al., 2005) (Figure 9.2). Thus, the hagfish appears to have neurohypophysial and hypothalamic hormones similar to those of other vertebrates.

Figure 9.2 A nearly mid-sagittal section through the neurohypophysis of the Atlantic hagfish, Myxine glutinosa, showing the accumulation of ir-GnRH in the dorsal wall of the neurohypophysis (arrowheads). This section was stained with anti-salmon GnRH. Inset, An enlargement of the rectangular area showing GnRH-positive neuronal cells. Arrows show GnRH-positive cell bodies. Scale bars: 100 μm; inset, 20 μm. (From Oshima, Y., K. Ominato and M. Nozaki. 2001. Distribution of GnRH-like immunoreactivity in the brain of lampreys and hagfish. Annual Activity Reports of the Sado Marine Biological Station, Niigata University, 31:4–5.)
At present, the adenohypophysis of the hagfish is the least understood of all the vertebrates. However, recent immunohistochemical studies provided the first clear-cut evidence for the presence of GTH and ACTH in the hagfish (Nozaki et al., 2005, 2007; Miki et al., 2006) (Figure 9.3). Although not conclusive, Nozaki et al. (2005) also suggested the presence of GH in the hagfish. In addition, these three adenohypophysial hormones were suggested to be the ancestral adenohypophysial hormones that have maintained their original functions throughout vertebrate evolution (Nozaki, 2008). On the other hand, the later derived hormones, such as prolactin and TSH, may have contributed to the expansion of vertebrates into new environments, as suggested by Kawauchi et al. (2002) and Kawauchi and Sower (2006) in the lamprey. Moreover, it has been further revealed that GTH cells, ACTH cells and unidentified cells that were assumed to include both undifferentiated cells and GH cells, were packed together in the same cell cluster of the hagfish adenohypophysis, and thus each cluster appeared to serve as a separate functional unit (Nozaki et al., 2007; Nozaki, 2008) (Figures 9.1e and 9.3). If the absence of the pars intermedia is the most ancestral vertebrate pituitary characteristic as found in the hagfish, MSH activity seems to be gained secondarily together with the differentiation of the pars intermedia. Further studies are needed to clarify this possibility.

**Figure 9.3** Three successive sagittal sections (a–c) through the adenohypophysis of an adult brown hagfish with developing gonads, stained with anti-ovine LHβ (oLHβ), biotin-conjugated *Lycopersicon esculentum* lectin (LEL), and anti-lamprey ACTH (lACTH), respectively. Boxes in a–c are shown enlarged in d–f, respectively. Asterisks in d show mass of cells, which are negative to anti-oLHβ, but are stained with LEL and anti-lamprey ACTH. These cells are assumed to be ACTH cells. Scale bars: (a–c) 200 μm; (d–f) 20 μm. (From Nozaki, M., T. Shimotani and K. Uchida. 2007. *Cell Tissue Res* 328:563–572. doi:10.1007/s00441-006-0349-3.)
Glycoprotein hormones (GPHs)

GTHs, in response to hypothalamic GnRH, are released from the pituitary and act on the gonads to regulate steroidogenesis and gametogenesis. Two GTHs, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), together with TSH form a family of pituitary hormones (Figure 9.4). They are heterodimeric glycoproteins consisting of two subunits, an α-subunit and a unique β-subunit. These glycoprotein hormones (GPHs) are believed to have evolved from a common ancestral molecule through duplication of β-subunit genes and subsequent divergence (Dayhoff, 1976;

Figure 9.4 Schematic diagram of the evolution of glycoprotein hormones in the hypothalamic – pituitary – gonadal axis. Ancestral thyrostimulin (α and β) existed before divergence of vertebrates. An ancestral thyrostimulin (α and β) diverged into GTH (α and β) and thyrostimulin (α and β) during the early phase of agnathan divergence. The GTH (α and β) formed a heterodimer in the pituitary and acted as the first adenohypophysial gonadotropic hormone during the evolution of agnathan species. This GTH dimer further diverged into three functional units of adenohypophysis, LH and FSH as two gonadotropins, and TSH as a thyrotropin, in the lineage to gnathostomes. (From Nozaki M. 2013. Front. Endocrinol. 4:200. doi:10.3389/fendo.2013.00200 with minor modification.)
Two GTHs have been identified in all taxonomic groups of gnathostomes, including actinopterygians (Kawauchi et al., 1989; Quérat et al., 2000), sarcopterygians (Quérat et al., 2004), and chondrichthyans (Quérat et al., 2001), but not in agnathans.

A single β-subunit of GTH was identified from the sea lamprey pituitary gland after extensive and exhaustive research that took over 20 years (Sower et al., 2006; Kawauchi and Sower, 2006). Using heterologous probes, there were many physiological and morphological evidence supporting the presence of a GTH in the lamprey (Larsen and Rothwell, 1972; Hardisty and Baker, 1982; Sower, 1998; Sower et al., 2006; Nozaki et al., 2008). However, despite extensive molecular and biochemical studies, the α-subunit of lamprey GTH was not identified which is now supported by synteny analysis of the genes from the lamprey genome (Sower et al., submitted). Instead, there is a thyrostimulin type alpha called A2 (see subsequent paragraph). It is suggested by these authors that the typical alpha subunit was lost in the lamprey lineage. It is now reported that lampreys have an ancestral non-classical, pituitary heterodimer GPH consisting of the thyrostimulin A2 subunit (GPA2) with the classical β subunit in the sea lamprey (Sower et al., submitted). The current hypothesis is that this one glycoprotein hormone may be acting as both a GTH and TSH hormone in some unknown differential manner (Sower et al., submitted).

Recently, a fourth heterodimeric GPH has been discovered in the human genome and termed “thyrostimulin” due to its thyroid-stimulating activity (Nakabayashi et al., 2002). The thyrostimulin α-subunit, called glycoprotein α-subunit 2 (GPA2), is homologous but not identical to the common α-subunit (GPHα or GPA1). With the discovery of GPA2 and glycoprotein β-subunit 5 (GPB5, thyrostimulin beta), homologs are present not only in other vertebrates, but also in invertebrates including fly, nematode and sea urchin (Sudo et al., 2005; Park et al., 2005), it is proposed that ancestral glycoprotein subunits existed before the divergence of vertebrates/invertebrates, and later gene duplication events in vertebrates produced the thyrostimulin (GPA2 and GPB5) and GTH/TSH [GPHα and GPHβ(LHβ/FSHβ/TSHβ)] (Sudo et al., 2005) (Figure 9.4). The basal lineage of chordates, such as tunicates and amphioxus, contains GPA2 and GPA5 in their genomes but not GPHα or GPHβ (Holland et al., 2008; Dos Santos et al., 2009, 2011; Tando and Kubokawa, 2009a, b). Lamprey also has GPA2 and GPB5 genes in addition to the canonical GTHβ (Sower et al., 2006, 2009; Dos Santos et al., 2009; Decatur et al., 2013, Sower et al., submitted). At present, no information is available as to the presence or absence of thyrostimulin GPA2/GPB5 in the hagfish.
Identification of hagfish GTH

A single GTH, which comprises $\alpha$- and $\beta$-subunits, was recently identified in the pituitary of the brown hagfish, *Paramyxine (=Eptatretus) atami*, one of the Pacific hagfish (Uchida et al., 2010) (Figure 9.4). Both subunits of GTH are produced in the same cells of the adenohypophysis, providing definitive evidence for the presence of a heterodimeric GTH in the hagfish. GTH increases at both the gene and protein levels corresponding to the reproductive stages of the hagfish (Figures 9.1c and d). Moreover, purified native GTH induces the concentrations of sex steroids (estradiol-17$\beta$ and testosterone) from cultured testis in a dose-dependent manner.

From the phylogenetic analysis, the hagfish GTH$\alpha$ forms a clade with the gnathostome GPH$\alpha$s. The hagfish GTH$\beta$ forms a clade with the TSH$\beta$s, however the bootstrap values are low and hagfishes evolved prior to the gnathostomes. The sea lamprey GTH$\beta$ also groups with the GPH$\beta$s but appears to be one of the outgroups of the LH$\beta$s. These results clearly show that the GTH identified in the hagfish acts as a functional GTH. From these data, the pituitary and its hormone early in vertebrate evolution are showing an intermediate stage as evident by only one GTH-like hormone in the agnathans versus the classical two GTHs found in gnathostomes (jawed vertebrates).

Now, it is quite clear that hagfish has a functional GTH in the pituitary. Then, a question arises as to the failure to detect any clear evidence for pituitary gonadotropic activity in the earlier study in hypophysectomized *Eptatretus stoutii* (Matty et al., 1976). In the study, gametogenesis appeared to be unaffected by hypophysectomy. On the other hand, in *Eptatretus burgeri*, the only hagfish known to have a definite breeding season (Ichikawa et al., 2000; Nozaki et al., 2000) (Figures 9.5 and 9.6), there have been some indications that gonadal development and spermatogenesis may be retarded after hypophysectomy (Patzner and Ichikawa, 1977). There is at least one possible explanation for the difference of results on hypophysectomy between two species. In the hagfish, the process of gametogenesis may be relatively autonomous, and may not be completely arrested by hypophysectomy. In both species, gametogenesis may still proceed, although at a slower pace, after hypophysectomy. If so, since *E. burgeri* exhibits clear seasonal changes in gonadal development, it is possible to detect the difference of the gonadal conditions between hypophysectomized and sham-control animals. On the other hand, in *E. stoutii*, developmental conditions of gonads vary among individuals, and thus it is difficult to detect clear difference between two groups. In support of this possibility, hypophysectomy experiments have been conducted extensively in lampreys, in which limited gonadal growth still continued after hypophysectomy (Evennett and Dodd, 1963; Larsen, 1973; Gorbman, 1983).
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Figure 9.5 (See color insert.) Annual growth curve of developing eggs of the female hagfish (Eptatretus burgeri) caught in three different locations: water of 6–10 m depths (closed circles), water of 50 m depth (open circles) and water of 100 m depth (triangles). Numbers of animals studied were 20–40 otherwise indicated in the figure. In most cases, ranges of the standard errors are within the size of the mean data points. Broken line indicates a regression line, which is made by the mean data points of all three locations. (From Nozaki, M., T. Ichikawa, K. Tsuneki and H. Kobayashi. 2000. Zool Sci 17:225–232.)

Figure 9.6 Annual changes in percentage of follicles containing each developmental stage of spermatogenic cells in the testis of hagfish (Eptatretus burgeri). Solid circles and bars indicate the mean and standard errors, respectively. Numbers indicate number of individuals studied. (From Nozaki, M., T. Ichikawa, K. Tsuneki and H. Kobayashi. 2000. Zool Sci 17:225–232.)
Feedback regulation of hagfish GTH functions by sex steroid hormones

Gonadal steroid hormones and hypothalamic hormones play major roles in controlling the synthesis and release of LH and FSH in gnathostomes (Figure 9.4). Both positive and negative feedback effects of gonadal steroids have been demonstrated in teleosts, depending on modes of administration and reproductive stages of animals. In general, in sexually mature fish, sex steroids are considered to regulate gonadal maturation and recrudescence, whereas in juvenile fish, sex steroids are considered to regulate puberty. Thus, negative feedback effects of estradiol and testosterone are evident during the latter stages of gonadal development; specifically, it has been shown that gonadal removal increases LH secretion in salmon (Larsen and Swanson, 1997), goldfish (Kobayashi and Stacey, 1990), and African catfish (Habibi et al., 1989). The observed increases in LH levels can be suppressed by the treatment with estradiol, testosterone or both. FSH is also controlled by steroid-dependent negative feedback loops in rainbow trout (Saligaut et al., 1998), salmon (Dickey and Swanson, 1998), and goldfish (Kobayashi et al., 2000). The negative feedback effects of steroids may be mediated primarily at the levels of the hypothalamic GnRH neurons (Vacher et al., 2002; Levavi-Sivan et al., 2006; Banerjee and Khan, 2008), because both in vivo and in vitro studies have shown that the expression of LHβ mRNA or FSHβ mRNA is often unchanged or increases following the exposure to estradiol, testosterone or both (Saligaut et al., 1998; Huggard-Nelson et al., 2002; Levavi-Sivan et al., 2006). However, in sexually immature teleosts, sex steroids appear to exert primarily a positive feedback effect that acts directly at the level of the pituitary and via effects on the GnRH system (Huggard-Nelson et al., 2002; Aroura et al., 2007). LH content and LH mRNA levels of the pituitary in juvenile fish increase in response to estrogens and aromatizable androgens (Huggard et al., 1996; Saligaut et al., 1998).

Estradiol treatment in the juvenile brown hagfish resulted in the marked accumulation of both immunoreactive (ir)-GTHα and ir-GTHβ in the pituitary (Nozaki et al., 2013). However, mRNA levels of GTHα and GTHβ in the pituitary were not, or only transiently, increased by the estradiol treatment (Nozaki et al., 2013). The latter results suggest that syntheses of both α- and β-subunits of GTH were not, or only transiently, affected by the estradiol treatment. Accordingly, the marked accumulation of both ir-GTH subunits could be attributed to the suppression of GTH secretion from the pituitary. From the study, it shows that the feedback effects of estradiol appeared to be inhibitory rather than stimulatory, and also mediated by the possible suppression of the secretion of GTH from the pituitary in these juvenile hagfish. These conditions in juvenile hagfish resembled to those in adults, but not in juveniles, of teleosts.
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(Saligaut et al., 1998; Huggard-Nelson et al., 2002; Levavi-Sivan et al., 2006). Such suppression of GTH secretion in the hagfish is probably regulated by the hypothalamic factors including GnRH, as mentioned below.

On the other hand, testosterone treatment in the juvenile brown hagfish had no effect on the staining intensities of the ir-GTHα and ir-GTHβ in the pituitary (Nozaki et al., 2013). Nevertheless, testosterone treatment resulted in the suppression of mRNA expressions of both GTHα and GTHβ in the pituitary (Nozaki et al., 2013). Therefore, testosterone probably acts to suppress both the synthesis and the secretion of GTH. This conclusion follows from the reasoning that if the secretion of GTH was not suppressed, the intensities of immunoreactions of both GTHα and GTHβ would have decreased due to decreased levels of mRNA expressions in both GTH subunits. Thus, it seems likely that estradiol and testosterone differ with regard to their roles in the regulation of synthesis and secretion of GTH in the pituitary of the hagfish.

Gonads and gonadal sex steroids

Gonads and germ cells

Reproductive organs and hormones of hagfishes have been mainly studied in four species of hagfish: Atlantic hagfish (Myxine glutinosa), Eastern Pacific Hagfish (E. stoutii) and Western Pacific hagfish (=Japanese hagfish) (E. burgeri and P. atami) (for reviews, see Hardisty, 1979; Gorbman, 1983; Patzner, 1998). Among these hagfishes, only E. burgeri live in shallower water less than 50 m in depth, and show a seasonal migration and a seasonal development of gonads(Kobayashi et al., 1972; Patzner and Ichikawa, 1977; Tsuneki et al., 1983; Ichikawa et al., 2000; Nozaki et al., 2000) (Figures 9.5 and 9.6). However, a subpopulation of E. burgeri is also found in water of greater than 50 m depth throughout the year, which exhibits seasonal development of gonads similar to that of migrating population (Ichikawa et al., 2000; Nozaki et al., 2000) (Figure 9.7).

The gonads of hagfishes are situated in the peritoneal cavity. The anlage for the ovary is found in the anterior part of the gonads, the one for the testis is in the posterior part close to the anus. The problem of sex differentiation in hagfishes was discussed in detail by Gorbman (1990), who concluded that gonadal differentiation in E. stoutii is juvenile progynous. The characteristic features of the hagfish gonads are the scanty number of mature eggs (less than about 40) and the small amount of sperm (Patzner, 1998). In M. glutinosa, most individuals are females or hermaphrodites, and males with no female tissue are rarely present. For example, 58% of M. glutinosa examined (n = 1080) only female gonadal tissue, 41% were hermaphrodites with both male and female tissue and 0.05% were males with no female tissue (Powell et al., 2004). On the other hand, the sex ratio of
the Pacific hagfish is nearly 1:1 *(E. stoutii, Conel, 1931; E. burgeri, Ichikawa et al., 2000; P. atami, Miki et al., 2006).*

**Plasma levels of sex steroid hormones in the hagfish gonads**

Sex steroids in vertebrate gonads have crucial roles in reproductive phenomena including sex differentiation, gametogenesis and gamete maturation. The physiological active gonadal steroids of higher vertebrates are progesterone from the corpus luteum, androgen from the testis and estrogen from the ovary.

In the hagfish, sex steroid hormones such as estradiol and testosterone have been detected in the circulating plasma (Matty et al., 1976; Weisbart et al., 1980; Schützinger et al., 1987; Nishiyama et al., 2013) or in the gonads (Hirose et al., 1975; Gorbman and Dickhoff, 1978; Powell et al., 2004, 2006), but their concentrations were very low near the lower limits of assay sensitivities. Among these studies, Schützinger et al. (1987) reported that plasma estrogen content increased in relation to the stages of ovarian development in female Atlantic hagfish, *M. glutinosa*. Powell et al. (2004, 2005) also reported using *in vitro* organ cultured ovaries that the number of females with large eggs increased following estradiol peaks in January in *M. glutinosa*. Nishiyama et al. (2013) further observed in *P. atami* that among estradiol-17β, testosterone and progesterone only plasma levels of estradiol-17β showed the significant correlation to ovarian development (Figure 9.7). In another study, Yu et al. (1981) demonstrated that the synthesis of hepatic vitellogenin was inducible by estrogens, estradiol and estrone, in *E. stoutii*. Based on these results, estrogenic control of ovarian development and hepatic vitellogenesis seems to have arisen early in vertebrate evolution.

In males, however, no clear relationships were observed between plasma estradiol or testosterone concentrations and testicular development, while plasma progesterone concentrations showed a significant inverse relationship with testicular development (Nishiyama et al., 2013) (Figure 9.7). The failure to correlate with the circulating levels of sex steroid hormones and gonadal developments in the male hagfish is discussed in relation to CYP11A mRNA expressions in the testis of the hagfish (see below).

**Expression of steroidogenic enzymes in the hagfish gonads**

The biosynthetic enzymes of sex steroids have been well studied in gnathostomes. Typically, there are three cytochrome P450 enzymes (CYP) such as P450 side chain cleavage (CYP11A), P450 17α-hydroxylase (CYP17) and P450 aromatase (CYP19) and two types of hydroxylated dehydrogenases (HSDs), such as 3β-HSD and 17β-HSD. Among these enzymes,
CYP11A is an enzyme that regulates the conversion from cholesterol to pregnenolone by its side chain cleavage activity, and it is the first and the essential enzyme of steroidogenesis.

Some information is available on the gonadal steroidogenesis in the hagfish (Patzner, 1998). Recently, based on EST analysis of the testis of the brown hagfish (*P. atami*), *CYP11A* was cloned (Nishiyama et al., 2015). Following the real-time PCR analysis, *CYP11A* mRNA expression levels were clearly correlated with the developmental stages of gonads.
in both sexes of the brown hagfish (Nishiyama et al., 2015) (Figure 9.8). These results are consistent with those in more advanced gnathostomes. For example, transcript for CYP11A in the gonads increased in correlation with gonadal development in both sexes of rainbow trout (Nakamura et al., 2005; Kusakabe et al., 2006) and female Japanese eel (Kazeto et al., 2006). Previously, it has been shown that plasma concentrations of estradiol-17β increased in correlation with the gonadal development in female brown hagfish (Nishiyama et al., 2013). Moreover, Yu et al. (1981) demonstrated that the synthesis of hepatic vitellogenin in E. stoutii was induced by estrogens, estradiol and estrone. Thus, CYP11A is suggested

![Figure 9.8](image.png)

**Figure 9.8** Relative CYP11A gene expressions in the gonads of female (a) and male (b) hagfish. The CYP11A mRNA levels were normalized by β-actin mRNA levels. Relative values are expressed as mean ± SE. Number in each column indicates number of animals studied. (a) J, juvenile ovary; N, non-vitellogenic adult ovary; E, early vitellogenic adult ovary; L, late vitellogenic adult ovary. (b) J, juvenile testis; S, adult testis with small GSI; M, adult testis with medium GSI; L, adult testis with large GSI. *P < 0.05; **P < 0.01; ***P < 0.001.
to play a crucial role in the synthesis of estradiol-17β, which in turn acts on ovarian development and hepatic vitellogenesis in the female hagfish.

In male hagfish, transcriptional levels of CYP11A increased in accordance with the developmental stages of testis, as well as in females (Nishiyama et al., 2015) (Figure 9.8). Moreover, in the testis incubated with hagfish GTH (5 μg/mL), CYP11A mRNA expression levels were significantly higher than those incubated without hagfish GTH (Nishiyama et al., 2015), indicating that gonadal CYP11A expression was induced by the pituitary GTH in the hagfish. However, as mentioned earlier, no relationship was obtained between the testicular development and plasma levels of estradiol or testosterone (Nishiyama et al., 2013).

Thus, there was a clear discrepancy between males and females in the relationship between the transcriptional levels of CYP11A and plasma steroid levels. These results clearly suggest a possibility that male hagfish uses other steroids than estradiol or testosterone as major androgens. In support of this possibility, recent studies in the lamprey have emphasized the importance of non-classical steroids, such as androstenedione and 15α-hydroxylated sex steroids (15α-hydroxytestosterone and 15α-hydroxyprogesterone) in serving as functional androgens (Lowartz et al., 2003; Young et al., 2007; Bryan et al., 2007, 2008). A receptor for androstenedione was recently described in the lamprey by Bryan et al. (2007). Since hagfish gonads also produce substantial amounts of unusual androgens, such as 6β-hydroxy testosterone and 5α-androstan-3β, 7α, 17β-triol, as well as androstenedione (Hirose et al., 1975; Kime et al., 1980; Kime and Hews, 1980), some of these steroids may act as functional androgens. Further study is required in order to clarify the role of these steroids in hagfish.

**Localization of steroidogenic cells in the hagfish gonads**

It is well established that sex steroid hormones are produced in the cells comprising the growing follicles (theca cells and granulosa cells) in the ovary of female gnathostomes. A two-cell type model has been proposed for the follicular steroidogenesis in the ovary (see Nagahama et al., 1994; Gore-Langton and Armstrong, 1998). In the model, steroid synthesis from cholesterol to androgen is performed in the theca cells, followed by the synthesis of estrogen from androgen in the granulosa cells.

By *in situ* hybridization, CYP11A mRNA signals were found in the theca cells of the ovary of *P. atami* (Nishiyama et al., 2015) (Figure 9.9), which is well accordance with those in gnathostomes. This result contrasted to the previous electron microscopy in the hagfish, in which cells, showing the characteristics associated with steroidogenesis, were not observed in the ovary of *M. glutinosa* (Fernholm, 1972) or *E. stoutii* (Tsuneki and Gorbman, 1977a). The reason for the difference of the results
was not clear, since Tsuneki and Gorbman (1977a) studied various structures of the hagfish ovary including those of large oocytes. Possibly, the theca cells of the hagfish ovary may not show typical ultrastructural features of steroidogenic cells.

It is also well established that sex steroid hormones are produced in the interstitial cells (Leydig cells) of the testis in male gnathostomes. For example, steroidogenic enzymes such as CYP11A, CYP17 and 3β-HSD were reported in the Leydig cells of the rainbow trout testis (Kobayashi et al., 1998). However, in some teleost species, such as pike and char, urodele amphibians and turtles, typical interstitial cells are absent, and a ring
of circumtubular cells (tubule-boundary cells) are considered to be the site of the production of sex steroid hormones (Gorbman and Bern, 1962). In the lamprey, Leydig cells in the testis are also suggested, in a histological study, to be steroid hormone-producing cells (Larsen, 1973, cited by Gorbman, 1983). In the hagfish, Tsuneki and Gorbman (1977b) described the ultrastructure of the testis of E. stoutii: they found no apparent steroidogenic cells until a body length of about 40 cm is attained. At that time, cells with the features of Leydig cells (e.g., smooth ER and tubular cristae) appeared among the spermatogenic follicles. In well agreement with Tsuneki and Gorbman (1977b), expression levels of CYP11A mRNA were very low in juveniles with their total length of less than 40 cm, but increased significantly in relation to testicular development in the brown hagfish (Nishiyama et al., submitted). Histological observations by in situ hybridization further revealed that CYP11A mRNA was expressed in both the Leydig cells and tubule-boundary cells of the developing testis (Nishiyama et al., submitted) (Figure 9.9). Thus, both types of cells, Leydig cells and tubule-boundary cells are considered the steroid-producing cells in the hagfish testis. It seems most likely that one of these two types of cells is adopted as the steroidogenic cells of the testis in gnathostomes.

Functional corpus luteum in the hagfish

Follicular atresia is a common feature in the hagfish ovaries (Gorbman, 1983), and it appears to be the method by which some 100 oocytes are reduced to approximately 30 that are grown and are ovulated. Using in vitro organ-cultured ovaries of M. glutinosa supplemented with pregnenolone, Powell et al. (2006) recently demonstrated that larger amounts of progesterone were released from atretic follicles (yellow bodies only) than from normal follicles. They hypothesized that hagfish possessed functional corpora lutea-like structures that produced progesterone. On the other hand, Nishiyama et al. (2013) reported in P. atami that plasma levels of sex steroid hormones including progesterone were significantly lower in adult females that possessed atretic follicles along with normal follicles they were in females that possessed only normal follicles (Figure 9.7), indicating reduced steroidogenic activity in females that possessed atretic follicles. Thus, a clear discrepancy is found on progesterone production of atretic hormones between Powell et al. (2006) and Nishiyama et al. (2013). Several possible explanations could be considered: (1) As Powell et al. (2006) pointed out that only specialized yellow bodies have potent steroidogenic activity, and thus most atretic follicles of P. atami do not have progesterone secreting activity. (2) Progesterone synthesis by the yellow bodies is suppressed under normal physiological conditions. (3) It is also possible that the progesterone released from the yellow bodies
Hypothalamic factors regulating the gonadotropic function of hagfish

GnRH

The synthesis and the secretion of GnRH are the key neuroendocrine function in the hypothalamic regulation of the HPG axis. To date, two to three isoforms of GnRH have been identified in representative species of all classes of gnathostomes and lampreys (Sower et al., 2009; Kavanaugh et al., 2008). GnRHs are also identified in tunicates (Adams et al., 2003), and several invertebrates belonging to lophotrochozoans (mollusk and annelid; Tsai and Zhang, 2008; Zhang et al., 2008), but not in the ecdysozoan lineages. On the other hand, adipokinetic hormone (AKH) has been identified as the ligand of the GnRH receptor of the insects, Drosophila and Bombyx (Staubli et al., 2002). An AKH – GnRH-like neuropeptide has been identified in the nematode Caenorhabditis elegans (Lindemans et al., 2009). A comparative and phylogenetic approach shows that the ecdysozoan AKHs, lophotrochozoan GnRHs and chordate GnRHs are structurally related, and suggested that they all originate from a common ancestor (Lindemans et al., 2011).

In the hagfish, GnRH has not yet been identified, but previous chromatographic and immunohistochemical studies suggested the presence of a GnRH-like molecule in the hypothalamic – neurohypophysial area (Braun et al., 1995; Sower et al., 1995; Kavanaugh et al., 2005). Sower et al. (1995) showed using the techniques of immunocytochemistry (ICC), HPLC and radioimmunoassay (RIA) with a specific lamprey GnRH-III antisera localized a lamprey-III GnRH-like molecule in the hypothalamus, adenohypophysis and the neurohypophysis of the Atlantic hagfish (M. glutinosa). In particular, a dense accumulation of GnRH-like immunoreaction was observed in the dorsal wall of the neurohypophysis with the use of antisera against chicken GnRH-II (GnRH2 type), salmon GnRH (GnRH3 type), lamprey GnRH-I (GnRH3 type) and lamprey GnRH-III (GnRH3 type) (Sower et al., 1995; Oshima et al., 2001) (Figure 9.2). Also reported in 1995, using six different antisera to GnRH (salmon PBL-49, lamprey 21–134, lamprey 1459, lamprey 1467, chicken-II and mammalian), ir-salmon GnRH-like molecule was shown to be present in the preoptic cells, hypothalamic infundibular nucleus, hypophysial stalk and distributed fibers in the brain of the Pacific hagfish, E. stoutii (Braun et al., 1995). The identity of an ir-salmon GnRH-like molecule supports that
latest information that type GnRH3 arose early in the vertebrate lineage (Decatur et al., 2013). Based on syntenic data, these authors proposed that there were four lineages of GnRH (GnRH1, 2, 3 and 4) that arose before the divergence of the ancestral agnathans and gnathostome lineages. GnRH4 was lost during these events. Lamprey GnRH-I and -III previously proposed to be part of a Group 4 are now considered to be part of the GnRH3 lineage (Decatur et al., 2013). Thus, it seems reasonable that hagfish may have retained a GnRH2 and/or GnRH3-like molecule.

In addition, Kavanaugh et al. (2005) reported seasonal changes in hypothalamic ir-GnRH in relation to gonadal reproductive stages in the Atlantic hagfish. Based on these investigations, it is likely hagfish indeed have a GnRH or GnRH-like peptide in the brain, although, the primary amino acid structure of GnRH has not been identified in these ancient fish. The primary structure of the GnRH (-like) molecules needs to be determined to confirm the presence of specific GnRH(s) in the hagfish brain. The presence and the location of a GnRH-like substance in the brain of the hagfish have led the authors to hypothesize that GnRH has a neuroendocrine function acting on the pituitary (Braun et al., 1995; Sower et al., 1995).

In vertebrates, the neuroendocrine axis has a central role in the control of reproduction by integrating internal and external cues during key developmental reproductive stages. As stated previously, GnRH is considered the major hypothalamic hormone orchestrating reproduction in all vertebrates. There is evidence as described in this chapter that the hypothalamic – pituitary axis emerged in the early ancestral vertebrates and that hagfish have certain conserved aspects of this complex neuroendocrine axis in the coordination and the integration of environmental and hormonal cues in controlling reproduction.

RFamide peptides

RFamide peptides play various important roles in the central nervous system in both invertebrates and vertebrates (Ukena and Tsutsui, 2005; Tsutsui and Ukena, 2006). Among RFamide peptides, PQRFamide peptide group and LPXRFamide (X = L or Q) peptide group share a highly conserved C-terminal Pro-Gln-Arg-Phe-NH2 motif (PQRFa motif), which are considered to be important for the interaction with their receptors, as well as the structure of their receptors showed high-sequence similarities (for reviews, see Ukena and Tsutsui, 2005; Tsutsui, 2009; Tsutsui et al., 2010). PQRFa peptides are mainly expressed in the spinal cord and medulla oblongata (Vilim et al., 1999; Liu et al., 2001), and act as neurotransmitters or neuromodulators in the opioid system in mammals (Roumy et al., 2007). They are also expressed in the hypothalamus (Vilim et al., 1999; Kalliomäki and Panula, 2004; Goncharuk et al., 2006) and have other functions, such as cardiovascular regulation (Panula et al., 1996),
neuroendocrine function(s) (Jhamandas and MacTavish, 2003; Jhamandas et al., 2006), and locomotor regulation (Kotlinska et al., 2007). LPXRFamide peptide group includes GTH-inhibitory hormone (GnIH) (see Tsutsui, 2009). GnIH was shown to be located in the hypothalamic–pituitary system and to decrease GTH secretion from the pituitary (Tsutsui, 2009; Tsutsui et al., 2010). However, studies on teleosts and amphibians have shown that functions of LPXRFa peptides were stimulatory or inhibitory (Koda et al., 2002; Ukena et al., 2003; Amano et al., 2006; Zhang et al., 2010; Shahjahan et al., 2011).

Recently, PQRFa peptides including LPQRFa were identified from the brains of sea lamprey (Osugi et al., 2006, 2012) and brown hagfish (Osugi et al., 2011). In the lamprey, LPQRFa peptide-positive neurons were localized in the hypothalamus, and their fibers were terminated in close proximity to GnRH-III neurons in addition to the neurohypophysis (Osugi et al., 2012). Moreover, intraperitoneal injection of LPQRFa stimulated the expression of lamprey GnRH-III in the hypothalamus and GTHβ mRNA expression in the pituitary (Osugi et al., 2012). Similarly, several PQRFamide peptides were identified in the brain of the brown hagfish (Osugi et al., 2011). Based on in situ hybridization and immunohistochemistry, hagfish PQRFamide peptide precursor mRNA and its translated peptides were localized in the infundibular nucleus of the hypothalamus. Dense immunoreactive fibers were found in the infundibular nucleus and some of them were terminated on blood vessels within the infundibular nucleus. Furthermore, LPQRFa peptide, one of the hagfish PQRFa peptides, significantly stimulated the expression of GTHβ mRNA in the cultured hagfish pituitary. The latter results clearly suggest that GTH functions of the hagfish pituitary are controlled by the hypothalamic factors.

**Hypothalamic – pituitary system of the hagfish**

Neither the hagfish nor lamprey has the anatomical equivalent of a median eminence to convey the neurohormones to the anterior pituitary. Most vertebrates except agnathans and teleost fish have a portal vascular system (median eminence) for transferring neurohormones from the hypothalamus to the adenohypophysis (Holmes and Ball, 1974; Gorbman et al., 1983). In teleosts, there is a direct innervation of the pars distalis in the anterior pituitary by neurosecretory neurons from the hypothalamus (Holmes and Ball, 1974; Gorbman et al., 1983). The agnathans do not have nervous or vascular communication between the brain and the pituitary (Holmes and Ball, 1974; Gorbman et al., 1983) (Figures 9.1a,b). It has been suggested the brain regulation of the pituitary in agnathans is achieved via diffusion. In lampreys, the authors concluded from their studies that neurosecretory peptides-like GnRH diffuse from the brain to the
adenohypophysis and thus regulate its secretory activity (Nozaki et al., 1994). Similarly it is generally considered that in hagfish, the hypothalamic factors, such as GnRH, reach the adenohypophysis simply by diffusion (Nozaki et al., 1975; Tsukahara et al., 1986; Gorbman, 1995). However, the dorsal wall of the hagfish neurohypophysis, where ir-GnRH nerve fibers and ir-AVT nerve fibers are terminated (Nozaki and Gorbman, 1983; Braun et al., 1995; Oshima et al., 2001) (Figure 9.2), is far from the adenohypophysis by the presence of the neurohypophysis itself. On the other hand, the blood vessels are richly distributed on the surface of the dorsal wall, and make the posterior hypophysial vascular plexus (Gorbman et al., 1963; Kobayashi and Uemura, 1972). Although most blood in the posterior hypophysial vascular plexus enter the posterior hypophysial vein of the anterior cardinal system, several small vessels proceed from the dorsal wall to the adenohypophysis in E. burgeri (Kobayashi and Uemura, 1972). These small vessels may contribute the regulation of the adenohypophysial functions. A pair of small blood vessels from the hypothalamus also enters the posterior hypophysial vascular plexus (Gorbman et al., 1963). These identified small blood vessels, along with some PQRFamide neuronal fibers terminating on the blood vessels within the hypothalamus (Osugi et al., 2011), suggest that further studies are needed to understand the anatomical relationship between the hypothalamus and the pituitary in hagfish. In an evolutionary sense, there are three different types of brain regulation of the pituitary that have developed in the vertebrates: the agnathan diffusional type; the teleostean direct innervational type and the vascular type seen in all other vertebrates (Nozaki et al., 1994; Gorbman, 1995). During evolution of the vertebrates, structural features of the pituitary and hypothalamus also evolved that perhaps optimized the communication between these tissues as vertebrates became larger and more complicated in form and distance between the hypothalamus and pituitary increased significantly (Gorbman, 1995). Unlike the lamprey with a diffusional type of brain regulation of the pituitary, the hagfish may represent an intermediate stage in the hypothalamic – pituitary anatomical relationship in vertebrates and may have both a diffusional and the beginnings of a “pre-median eminence”.

Conclusion

The pituitary gland and all major adenohypophysial hormones were major evolutionarily events that emerged prior to or during the differentiation of the ancestral jawless vertebrates (agnathans). The acquisition of the pituitary gland in vertebrates along with the differentiation of the hypothalamus has led to physiological divergence, including reproduction, growth, metabolism, stress and osmoregulation in subsequent evolution of jawed vertebrates. Since hagfish represent the most basal and
primitive vertebrate that diverged over 550 million years ago (Janvier, 1996), they are of particular importance in understanding the evolution of the HPG axis related to vertebrate reproduction. Recent studies clearly show that the hagfish have a conserved, functional HPG axis similar to that of more advanced gnathostomes. However, there are distinct differences in both lamprey and hagfish HPG axes compared with later evolved differences suggesting an intermediate stage in development of the pituitary and its hormones. An understanding of the evolutionary events in these jawless vertebrates is critical in our understanding of the evolution of the HPG axis. We propose that this HPG system likely evolved from an ancestral, pre-vertebrate exclusively neuroendocrine mechanism by gradual emergence of components of a new control level, the pituitary gland and can provide important clues for understanding the organization of the hypothalamus and pituitary as essential regulatory systems in all vertebrates.

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References
Chapter nine: Hypothalamic – pituitary – gonadal endocrine system


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