DISSERTATION FOR THE DOCTORAL DEGREE

Studies on the regulation of kisspeptin/gonadotropin-inhibitory hormone system in the lunar-synchronized spawning of grass puffer

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Contents

Chapter 1

General introduction

1.1. Hypothalamic–pituitary–gonadal axis in fish

Reproduction is controlled by the hypothalamic–pituitary–gonadal (HPG) axis in vertebrates. The HPG axis receives internal and external signals that are related to reproductive functions, and it integrates these signals to transmit into peripheral organs as reproductive output. The HPG axis is generally conserved from agnathans to humans and plays a major role in the control of reproduction through coordinated regulation of the hypothalamus and the pituitary.

1.1.1. Anatomical basis of the hypothalamus-pituitary system

The hypothalamus is a vital part of the brain that acts as a major homeostatic regulator for wide range of physiological process. The hypothalamus basically integrates various sensory inputs from the local neuronal afferents as well as peripheral circulatory systems. Vertebrate hypothalamus shows similar anatomical organization which divided into four divisions: preoptic, anterior, tuberal and mammillary hypothalamus from rostral to caudal and each of which has a lateral, medial, and (medial-most) periventricular zone (Burbridge et al. 2016).

In fish, hypothalamic cells have been characterized by sending axons into the pituitary using chemical (Kah et al., 1983) and electrolytic lesions (Kah et al., 1987). The pituitary is a major gland of the endocrine system that controls various physiological functions around the body. The pituitary is attached to the hypothalamus by a short stalk (infundibulum) that emerges from the ventral surface of the central region of the hypothalamus in almost all vertebrates including fish. These stalks consist of neurosecretory fibers originating from the hypothalamus and project directly to the pituitary. The pituitary gland is grossly divided into the adenohypophysis and the neurohypophysis. The adenohypophysis contains different cells secreting a variety of pituitary hormones and thus represents the glandular part

of the pituitary (Fig. 1.1). On the other hand, the neurohypophysis part of the tetrapod pituitary comprised of nerve fibers which are initiated from the hypothalamus and secrete neurohypophysial hormones into the general circulation, although this part is closely allied with the pars intermedia in fish and often connected with neurointermediate lobe (Nagpal et al., 2019).

Anatomically different neurosecretory cells in the hypothalamus play vital role for various hypophysiotropic functions. These cells connect with different targets in the adenohypophysis, secreting different neurohormones, which serve particular functions through the hypothalamic-pituitary system.

1.1.2. HPG axis in the control of reproduction

One of the most important functions of the hypothalamic-pituitary system is to regulate reproduction. Several neurohormones such as gonadotropin-releasing hormone (GnRH), kisspeptin and gonadotropin-inhibitory hormone (GnIH) are produced from the hypothalamus and stimulate or inhibit the synthesis and release of two gonadotropins (GTHs), namely, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the pituitary. Later, these GTHs stimulate gonadal maturation and the secretion of sex steroid hormones such as androgens, estrogens and progestins. These sex steroids can affect the activity of the hypothalamic-pituitary system through feedback action (Fig. 1.2). This overall structure of the HPG axis is shared in vertebrates, although the hormones in this axis are structurally and functionally different depending on animal groups and species to facilitate reproduction and sexual behaviors in their own manners. In fish, two other pituitary hormones, growth hormone (GH) and prolactin (PRL) are also involved in the regulation of reproduction under control of GnRH and GnIH as described in the next section.

1.1.3. Pituitary hormones (GTHs, GH and PRL)

It is well established that fish reproduction is controlled by FSH and LH released from the pituitary (Yaron et al., 2003; Zohar et al., 2010). These two hormones are heterodimers which consist of a common glycoprotein α (GPα) subunit and a hormone–specific β subunit

(FSHβ or LHβ) (Pierce and Parsons, 1981). Numerous studies have reported the physiological roles of FSH and LH and their regulation in a variety of fish species (Yaron et al., 2003; Ando and Urano, 2010). Most studies on GTH functions have been focused on their expression profiles during reproductive cycles at the transcript and/or protein levels (Swanson et al., 2003) and the effects on gonadal steroidogenesis (Luckenbach et al., 2010; Chauvigne et al., 2012), vitellogenesis (Tyler et al., 1991) and on final gamete maturation (Nagahama et al., 2008; Sreenivasulu et al., 2009). FSH is mostly involved in promoting early gonadal development and growth, whereas LH plays an important role in regulating the late stage of gametogenesis, including the final gamete maturation and release (ovulation and spermiation) (Ogiwara et al., 2013; Chauvigne et al., 2014).

GH along with GTHs takes a noticeable part in the teleost gonadal development (Canosa et al., 2007). GH has been found to play an important role in gametogenesis (Loir, 1999) and steroidogenesis (Singh et al., 1988). In addition, GH increases the gonadal steroidogenic response to LH (Van der Kraak et al., 1990). The expression of GH gene has been shown to be responsive to estradiol-17β (E2) and GnRH (Trudeau et al., 1992; Zou et al., 1997; Klausen et al., 2002; Ando et al., 2004; Onuma et al., 2005). On the other hand, PRL is also involved in reproductive function but also in various other biological activities in vertebrates (Whittington and Wilson, 2013). In fish, PRL stimulates E2 production in guppy (Tan et al., 1988) and testosterone (T) production in tilapia (Rubin and Specker, 1992). PRL gene expression is responsive to E2 and GnRH (Bhandari et al., 2003; Cavaco et al., 2003; Onuma et al., 2005). Moreover, studies on GnIH in various vertebrates including fish has shown that GnIH plays as a multifunctional hypophysiotropic factor regulating the expression of GH and PRL as well as GTHs (Koda et al., 2002; Ukena et al., 2003; Amano et al., 2006; Moussavi et al., 2014; Shahjahan et al. 2016).

1.1.4. GnRH

GnRH has long been regarded as a pivotal hypothalamic neurohormone in the control of reproduction in vertebrates. It was first isolated as a luteinizing hormone-releasing factor from pigs and sheep (Amoss et al., 1971; Matsuo et al., 1971) and later named GnRH due to

its ability to stimulate the secretion of both LH and FSH. Among teleost fish, GnRH was first identified in salmon (Sherwood et al., 1983) following that two or three forms of GnRHs exist within the same species (Okubo and Nagahama, 2008). There are three main groups in GnRH derivatives in fish. First group is referred to as GnRH1, mainly expressed in the preoptic area (POA) in various fish species. Second group is referred to as GnRH2, formerly as chicken GnRH-II, present from cartilaginous fish to mammals. GnRH2 is consistently expressed in the midbrain tegmental area of these vertebrates. The third group, referred to as GnRH3, contains only salmon GnRH in some groups of fish. GnRH3 is mainly expressed in the rostral part of forebrain, particularly in the olfactory region and the terminal nerve ganglion. GnRH1 is a hypophysiotropic neurohormone, stimulating the secretion of FSH and LH from the pituitary, while GnRH2 and GnRH3 plays as a neuromodulator in behaviors associated with reproduction (Oka, 2009).

1.2. Kisspeptin and kisspeptin receptor

Kisspeptin is a neuropeptide that belongs to the RFamide (Arg-Phe-NH_2) peptide family and a product of the *Kiss1* gene. Previously, kisspeptin was named metastin since it inhibits tumor metastasis in mammals (Lee et al., 1996). It is now established in mammals that kisspeptin is involved in the regulation of reproduction through stimulation of GnRH secretion. Mature kisspeptin peptides in mammals are cleaved into endogenous fragments such as Kp54, Kp16, Kp14, Kp13 and Kp10 (Beltramo et. al. 2014). The C-terminus decapeptide Kp10 (Kiss-10) is the minimum active site and allows them to bind to G-protein coupled receptor 54 (GPR54) or Kiss1r (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001).

1.2.1. Kisspeptin in fish

In fish, there are two paralogous genes for kisspeptin (*kiss1* and *kiss2*) (Felip et al., 2009; Saha et al. 2016), although some fish species including grass puffer and tilapia have only one gene, *kiss2* (Shahjahan et al., 2010; Ogawa et al., 2013). In fish, kisspeptin has been shown to play an important role in reproduction like mammals, but two isoforms, Kiss1 and Kiss2,

differentially regulate the HPG axis. Several in vivo studies have shown that the administration of Kiss2 stimulated the secretion of FSH and LH in sea bass (Felip et al., 2009; Espigares et al. 2015), the expression of *fshb* and *lhb* in chub mackerel (Ohga et al. 2014) and the expression of *lhb* and *gnrh1* in tilapia (Park et al. 2016), whereas Kiss1 stimulated the secretion of LH in goldfish (Li et al., 2009) and FSH in striped bass (Zomora et al. 2014). In the grass puffer, the levels of *kiss2* expression were higher in the brain during breeding season when compared with non-breeding season (Shahjahan et al., 2010).

In the brain, the habenula nucleus mainly contains Kiss1 neurons (Kanda et al. 2008; Kitahashi et al., 2009; Servili et al. 2011; Wang et al. 2013; Ogawa et al. 2014; Song et al. 2015) along with the expression in the POA (Kanda et al. 2008; Kitahashi et al. 2009; Wang et al. 2013). Kiss1 neurons in the nucleus ventral tuberis express sexual dimorphism with increased number in the males of medaka (Kanda et al., 2008). Kiss2 neurons are largely distributed in the POA, thalamus, ventral and caudal hypothalamus (Servili et al. 2011; Kanda et al. 2012; Escobar et al. 2013; Ando et al. 2014; Song et al. 2015).

1.2.2. Kisspeptin receptor in fish

So far, four subtypes of kisspeptin receptor (Kiss1r, Kiss2r, Kiss3r, and Kiss4r) have been identified in non-mammalian vertebrates and there are two subtypes (Kissr1 and Kissr2) in fish (Pasquier et al. 2014). Piscine kisspeptin system was first reported in tilapia showing an expression of kisspeptin receptor in GnRH neurons (Parhar et al., 2004). Following this, kisspeptin receptor cDNA has been isolated from several fish species including gray mullet (Nocillado et al., 2007), cobia (Mohammed et al., 2007), fathead minnow (Filby et al., 2008), zebrafish (van Aerle et al., 2008), goldfish (Li et al. 2009), grass puffer (Shahjahan et al., 2010), cichlid fish (Grone et al. 2010), striped bass (Zmora et al. 2012) and medaka (Kanda et al. 2013).

So far, few works have been done on detail neuroanatomical distribution of kisspeptin receptor types in teleosts. *kiss1r* and *kiss2r* mRNAs were localized in the brain by in situ hybridization (Grone et al., 2010; Servili et al., 2011; Kanda et al., 2013) and laser-capture microdissection coupled with real-time PCR techniques (Ogawa et al., 2012). *kiss1r*- and

kiss2r-expressing neurons in the POA and hypothalamus and their co-localization and close proximity with GnRH and isotocin neurons suggests that kisspeptin is involved in the regulation of reproduction and homeostasis and instinctive behaviors. In the zebrafish, *kissr1* mRNA are predominantly expressed in the habenula and also observed in the dorsal interpeduncular nucleus, which receives innervations of the habenula Kiss1-immunoreactive (ir) fibers (Ogawa et al., 2010, 2012; Servili et al., 2011). On the other hand, *kiss2r* mRNA are widely expressed in the fish brain, which includes the olfactory bulb, telencephalon, POA, midbrain, hypothalamic nuclei, cerebellum and the spinal cord (Grone et al., 2010; Servili et al., 2011; Ogawa et al., 2012).

1.3. GnIH (LPXRFamide peptide) and GnIH receptor

GnIH is a neuropeptide that belongs to the RFamide peptide family like kisspeptin and forms the LPXRFamide (LPXRFa) peptide subfamily (Kriegsfeld et al., 2006; Tsutsui and Ukena, 2006; Tsutsui et al., 2007). GnIH was first identified in quail as an inhibitor of LH secretion (Tsutsui et al., 2000). Since the first identification of fish GnIH in goldfish (Sawada et al., 2002), studies on GnIH orthologs in teleost have attracted much attention. It has become more obvious that GnIH plays an important role in reproduction through directly acting on the pituitary, stimulating or inhibiting GTHs, GH and PRL (Tsutsui et al., 2010a; Ogawa and Parhar, 2014; Shahjahan et al., 2016). The action of GnIH is mediated by GnIH receptor (GnIH-R), which is also a G-protein coupled receptor, GPR147.

1.3.1. GnIH in fish

In fish, GnIH orthologs have been identified in a variety of fish species such as goldfish (Sawada et al., 2002), sockeye salmon (Amano et al., 2006), zebrafish (Zhang et al., 2010), grass puffer (Shahjahan et al., 2011) and tilapia (Biran et al, 2014), and most of the teleost GnIH precursors encode two or three GnIH or GnIH-related peptides. Besides mammals, where GnIH shows stimulatory and inhibitory effects on LH release depending on reproductive conditions particularly in seasonal breeding animals (Ubuka et al., 2012), in fish, the effects of GnIH on GTH synthesis and release are both stimulatory and inhibitory

depending on reproductive stage and species (Amano et al., 2006; Zhang et al., 2010; Moussavi et al., 2012; Qi et al., 2013; Biran et al., 2014). In addition, GnIH shows inhibitory effects on basal and GnRH-induced GH release from the goldfish pituitary but these effects are seasonally dependent (Moussavi et al., 2014). In the grass puffer, the goldfish LPXRFa peptide-1 stimulated the expression of *fshb* and *lhb* (Shahjahan et al., 2011) and *gh* and *prl* (Shahjahan et al., 2016) in the cultured pituitary cells.

In the brain, GnIH neurons were mainly found in the posterior periventricular nucleus (NPPv) in the hypothalamus (Sawada et al., 2002; Amano et al., 2006; Biswas et al., 2015; Ogawa et al., 2016; Paullada-Salmerón et al., 2016a;) and in some species expression was extended in other brain regions (Sawada et al., 2002; Biswas et al., 2014; Paullada-Salmerón et al., 2015; Di Yorio *e*t al., 2016).

1.3.2. GnIH-R in fish

The presence of *gnihr* transcripts has been determined in the brain and pituitary of some fish species (Zhang et al. 2010; Shahjahan et al. 2011) by RT-PCR. However, the detailed neuroanatomical characterization of GnIH-R has been limited in fish brain due to the lack of specific antibodies against fish GnIH-R. In the brain of tilapia, GnIH-R-ir neurons were widely distributed in the olfactory bulb, ventral/dorsal telencephalon, POA, hypothalamus, optic tectum, semicircular torus and caudal midbrain tegmentum. GnIH-R-ir cells were also extended to the pituitary level (Ogawa et al. 2016). In the brain of goldfish, three subtypes of GnIH-R genes were expressed in the POA, NPPv and lateral tuberal nucleus and two GnIH-R subtype genes were expressed in the pars intermedia of the pituitary (Qi et al. 2013).

1.4. Periodic regulation of reproduction in fish (seasonal, monthly and daily regulation)

Linkage of reproduction to the periodicity of environmental cues is a recognized adaptive feature in the life cycles of most vertebrate species. Time and light serve as a major environmental cue used to synchronize seasonal, monthly and daily changes in reproductive functions. For marine organisms, water temperature and tidal change are also used for their synchronized reproduction and sexual behavior. Many organisms use combined actions of

different environmental signals to synchronize their reproductive activities, and these signals are considered to be accompanied by integration of the HPG axis, particularly, GnRH, kisspeptin and GnIH system in the hypothalamus that are the most significant regulator of reproduction.

In fish, seasonally variable environmental cues such as light and water temperature are considered to regulate the GnRH/kisspeptin/GnIH system according to a variety of reproductive cycles. For the regulation by light, melatonin, the hormone produced from the pineal gland, is involved in transmitting the information of changes in light intensity and photoperiod (day length) to the hypothalamus. The secretion of melatonin is under control of time and light, being high in the nighttime and low during the daytime (Reiter, 1993). Thus, melatonin, “the nocturnal hormone” can transmit the seasonal changes in photoperiodic information to the central and peripheral organs (Falcón et al., 2007). In birds and mammals, melatonin has been shown to regulate GnIH expression and thus participates in the neuroendocrine control of seasonal reproduction (Ubuka et al., 2005; Revel et al., 2008).

On the other hand, studies have showed that seasonal fluctuations in water temperature have direct influences on fish reproduction such as gonadal maturation, ovulation and successive fertility, affecting the expression of GTHs (King et al., 2007; Soria et al., 2008; Wang et al., 2010; Arantes et al., 2011; Hermelink et al., 2011). However, the molecular and neuroendocrine mechanisms underlying the regulation of reproduction by water temperature are poorly understood. In some fish species, the expression of GnRH genes has been shown to be suppressed by anomalous increase and decrease in water temperature (David and Degani, 2011; Levy et al., 2011; Okuzawa and Gen, 2013). In zebrafish, decreased expressions of *kiss2* as well as *gnrh3* were observed in fish exposed to low and high temperature conditions (Shahjahan et al., 2013).

Another important periodic characteristic of marine organisms is lunar- and/or tidal cycle-synchronized reproduction, seen in a wide variety of organisms such as corals (Harrison et al., 1984), insects (Kaiser et al., 2011) and teleost fishes (Takemura et al., 2004b). In these organisms, changes in moonlight and tide are considered to be main environmental cues that entrain biological clocks that are connected to lunar and tidal cycles, i.e. circalunar

and circatidal clocks (Leatherland et al., 1992; Takemura et al., 2004b). However, there is a paucity of information on the molecular mechanisms of the lunar/tidal cycle-synchronized biological clocks and how these clocks regulate to the HPG axis. Several studies have tried to identify signal molecules that are linked to the lunar/tidal cycle information and reproduction. In the golden rabbitfish, which spawns around the first quarter moon, the plasma levels of melatonin at midnight are higher on the day of new moon than full moon. This lunar phase-dependent variation in the plasma melatonin concentrations at night has been considered to be critical for the occurrence of the lunar-synchronized spawning in the golden rabbitfish (Takemura et al., 2004b). However, it is still unknown how melatonin regulates the GnRH/kisspeptin/GnIH system in fish.

1.5. Grass puffer as a model animal for study on the GnRH/kisspeptin/GnIH system

The grass puffer, *Takifugu alboplumbeus*, is a common intertidal puffer species in Japan which shows unique reproductive physiology. In early summer from May to July, they aggregate at certain seashore place for spawning 2–3 h before high tide at dusk only during spring tide every two weeks, and then spawning occurs 1–2 h before high tide during flood tide (Motohashi et al., 2010; Ando et al., 2013). Therefore, spawning of the grass puffer is tightly connected with seasonal, lunar and tidal rhythms as well as daily rhythm where environmental factors such as light, tide, and water temperature play an important role in the control of reproduction (Ando et al., 2013). Furthermore, according to previously known time and place of the spawning, it can be easily obtained spawning fish as well as non-spawning fish that usually subsist in the coastal area. Taken together with these interesting facts, the grass puffer provides an excellent animal model for studying the molecular and neuroendocrine mechanisms of the periodic regulation of reproduction.

Previous studies on the grass puffer have facilitated this species to be an excellent experimental model for semilunar (two-week cycle) spawning rhythm. Three types of GnRH genes showed differential expression patterns during reproductive cycle in the grass puffer (Shahjahan et al., 2010a), where the expressions of *gnrh1* and *gnrh3* were substantially elevated during the breeding season along with the considerable increase in the levels of GTH

subunit mRNAs and plasma sex steroids (Shahjahan et al., 2010; Ando et al., 2013). For the genes encoding kisspeptin (*kiss2*), kisspeptin receptor (*kiss2r*), GnIH (*gnih*) and GnIH-R (*gnih-r),* their expression levels also increased during the breeding season (Shahjahan et al., 2010b; Shahjahan et al., 2011). Interestingly, they also showed daily and circadian oscillations during the spawning season, suggesting that the expression of kisspeptin, GnIH and their receptor genes may be regulated by melatonin and circadian clock (Shahjahan et al., 2010b; 2011; Ando et al., 2014). Moreover, GnIH (goldfish LPXRFa peptide-1) was found to stimulate the expression of *fshb* and *lhb* (Shahjahan et al., 2011) and *gh* and *prl* (Shahjahan et al., 2016) in the cultured pituitary cells of mature fish. These results indicate that both kisspeptin and GnIH may have stimulatory roles in the control of reproduction in the grass puffer, and thus their periodical regulation is most important point to clarify the molecular mechanism of the seasonal, semilunar, and precisely timed spawning.

1.6. The purpose of the present study

Based on our previous studies on the GnRH/kisspeptin/GnIH system in the grass puffer, its periodical regulation, possibly by melatonin, biological clock and temperature, is the most important point to clarify the molecular neuroendocrine mechanism in the control of the semilunar spawning in this fish. So, in this study we first established neuroanatomical basis of kisspeptin and GnIH neurons to understand its functional role, and then examined its periodic regulation with special reference to seasonal (temperature) and monthly (lunar-age dependent) regulation. For temperature regulation, Shahjahan et al. (2017) recently reported that anomalous temperature was proved to inhibit the expressions of *kiss2*, *kiss2r*, *gnrh1*, *fshb* and *lhb* along with gonadal maturation during the breeding season. Therefore, temperature regulation of *gnih*, *gnih-r*, *gh* and *prl* was examined in this study.

Accordingly, the present study was undertaken to clarify the following four points:

1. To determine the neuroanatomical distribution of kisspeptin- and kisspeptin receptor-ir cells in the brain of grass puffer;
2. To determine the neuroanatomical distribution of GnIH- and GnIH-R-ir cells in the brain of grass puffer;

3. To determine the regulation of *gnih*, *gnih-r*, *gh* and *prl* expressions by water temperature;
4. To clarify lunar-age dependent oscillations in expression of *kiss2*, *kiss2r*, *gnih* and gnihr during a lunar month in the breeding season.

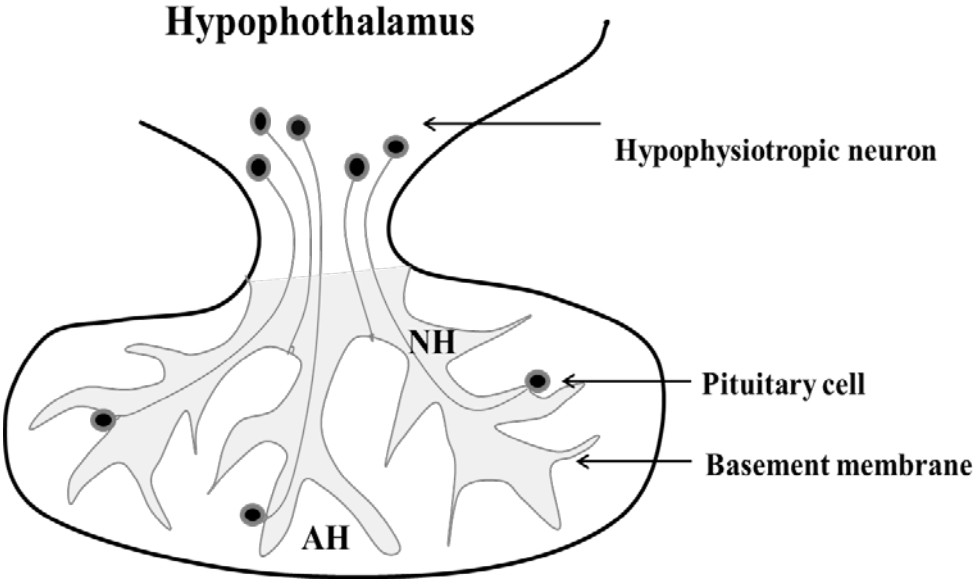


Fig. 1.1. Schematic representation of a lateral view of teleost pituitary showing hypophysiotropic neurons and adenohypophyseal cells. The hypophysiotropic neurons innervate the basement membrane separating the adenohypophysis (AH) from the neurohypophysis (NH) or directly at adenohypophyseal cells.

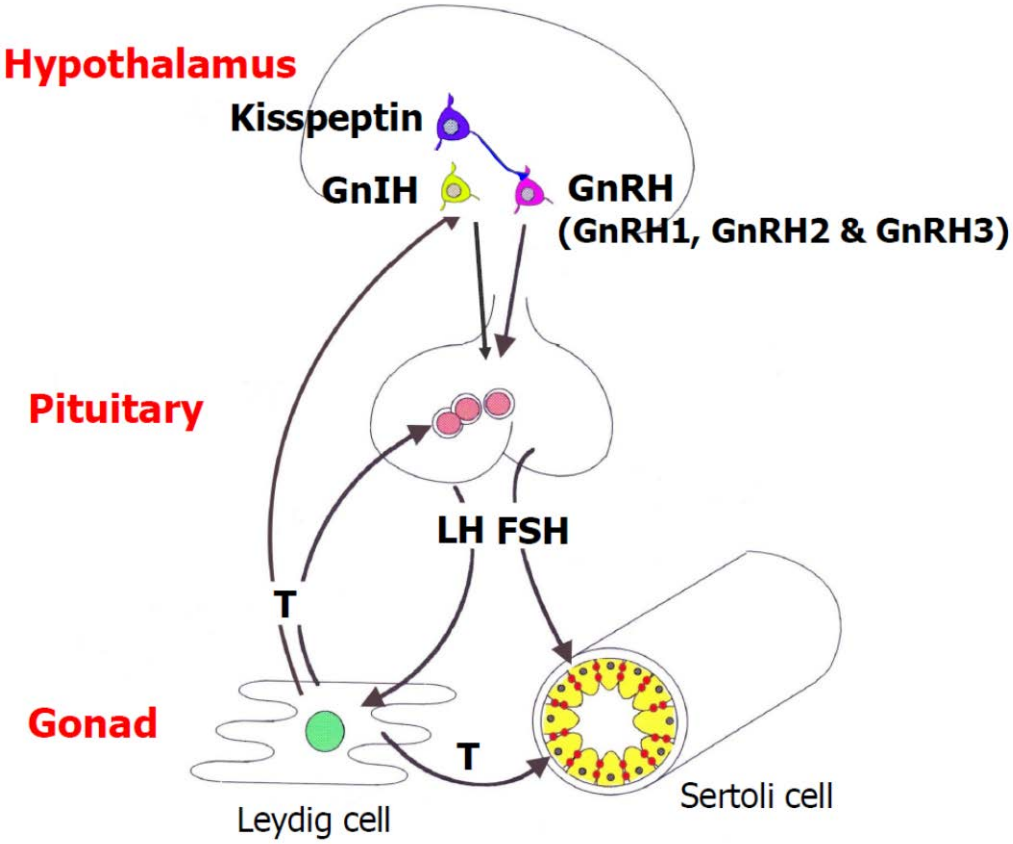


Fig. 1.2. Schematic representation of the hypothalamic–pituitary–gonadal axis regulating reproduction in vertebrates. GnRH, gonadotropin-releasing hormone; GnIH, gonadotropin-inhibitory hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; T, testosterone.

Chapter 2

Neuroanatomical studies on the kisspeptin and kisspeptin receptor system

2.1. Introduction

Kisspeptin is a neuropeptide that belongs to the RFamide (Arg-Phe-NH_2) peptide family and a product of the Kiss1 gene. It is established in mammals that kisspeptin is involved in the regulation of reproduction through stimulation of gonadotropin-releasing hormone (GnRH) secretion. Neuroanatomical studies have showed that kisspeptin-immunoreactive (ir) fibers are closely associated with GnRH neurons, and the colocalization of GnRH neurons with kisspeptin receptors, which are G-protein coupled receptor, GPR54, provides the structural and functional bases of the role of kisspeptin system as a central regulator of reproduction in mammals (Kinoshita et al., 2005; Clarkson and Herbison, 2006).

In fish, there are two paralogous genes for kisspeptin (*kiss1* and *kiss2*) (Kanda et al. 2008; van Aerle et al. 2008; Felip et al. 2009; Kitahashi et al. 2009; Lee et al. 2009; Li et al. 2009; Zmora et al. 2012; Saha et al. 2016). However, some percomorph species have only one gene, *kiss2* (Shahjahan et al., 2010b; Mechaly et al., 2011; Tena-Sempere et al., 2012; Ogawa et al. 2013). It has been shown that the regulation of the hypothalamic–pituitary–gonadal (HPG) axis by two forms of kisspeptin are different among species. For example, the administration of Kiss1 increased the plasma luteinizing hormone (LH) levels in goldfish (Li et al., 2009) and stimulated spermiation in chub mackerel (Selvaraj et al. 2013). Kiss1 was found to regulate the serotonergic system in zebrafish (Ogawa et al. 2012). On the other hand, Kiss2 stimulated the secretion of LH and follicle-stimulating hormone (FSH) in several fish species (Felip et al. 2009; Kitahashi et al. 2009; Ohga et al. 2014; Espigares et al. 2015; Park et al. 2016). In striped bass, Kiss1 showed stimulatory but Kiss2 showed inhibitory effects on the expression of *lhb* and *fshb* (Zmora et al. 2014). Furthermore, recently, gene knockout study for kisspeptin (*kiss1* and *kiss2*) and its receptor (*kissr1* and *kissr2*) in zebrafish demonstrated

that mutant lines for both ligand and receptor genes showed normal reproductive capability, suggesting that kisspeptin signaling is dispensable for reproduction (Tang et al., 2014). In addition, the lack of expression of kisspeptin receptor in GnRH neurons in medaka (Kanda et al., 2013) and European sea bass (Escobar et al., 2013) further supported this notion.

These differential and controversial roles of kisspeptin argue to elucidate neuroanatomical distribution of kisspeptin and kisspeptin receptor. Previous studies revealed that Kiss1 and Kiss2 were distinctly distributed throughout the brain. Most of the experiments were done using in situ hybridization (ISH) due to the difficulty of antibody production. *kiss1*-expressing neurons were mainly localized in the habenular nucleus (Kanda et al. 2008; Kitahashi et al., 2009; Servili et al. 2011; Ogawa et al. 2012; Song et al. 2015; Ohga et al. 2017) and in the preoptic area (POA) (Kanda et al. 2008; Kitahashi et al. 2009). On the other hand, *kiss2*-expressing neurons are widely localized in the POA, thalamus, ventral and caudal hypothalamus and the mesencephalic area (Servili et al. 2011; Kanda et al. 2012; Escobar et al. 2013; Ando et al. 2014; Song et al. 2015). Moreover, a study using immunohistochemistry (IHC) in zebrafish showed that Kiss2-ir fibers profusely innervate the ventral forebrain and made close apposition with GnRH3-ir neurons (Servili et al. 2011).

For the brain localization of kisspeptin receptor, several studies have tried to identify *kissr*-expressing cells in the brain by laser-capture microdissection coupled with real-time PCR (Ogawa et al., 2012) and ISH (Grone et al., 2010; Servili et al., 2011; Kanda et al., 2013; Ando et al., 2014). In the zebrafish, *kissr1* mRNA are predominantly expressed in the habenula and in the dorsal interpeduncular nucleus, which receives innervations of the habenula Kiss1-ir fibers (Ogawa et al., 2010, 2012; Servili et al., 2011). On the other hand, *kiss2r* mRNA are widely expressed in the fish brain, which includes the olfactory bulb, telencephalon, POA, midbrain, hypothalamic nuclei, cerebellum and spinal cord (Grone et al., 2010; Servili et al., 2011; Ogawa et al., 2012; Ando et al., 2014). The co-expression of *kissr* mRNA in *gnrh1*-expressing neurons has been shown in several cichlid fish, supporting the role of kisspeptin in the regulation of reproduction (Parhar et al., 2004, Grone et al., 2010; Zmora et al., 2012). However, in medaka, the lack of expression of *kissr* in GnRH neurons

and colocalization of *kissr* mRNA with isotocin in the magnocellular preoptic nucleus suggests non-reproductive role of kisspeptin (Kanda et al., 2013).

The present study aims to clarify neuroanatomical structure of the kisspeptin system in the grass puffer (*Takifugu alboplumbeus*). The grass puffer is an intertidal puffer species, having an interesting and unique spawning behavior that is synchronized with lunar and tidal cycles (Motohashi et al., 2010; Ando et al., 2013). In early summer from May to July, they aggregate at certain seashore place for spawning 2–3 h before high tide at dusk only during spring tide every two weeks, and then spawning occurs 1–2 h before high tide during flood tide (Motohashi et al., 2010; Ando et al., 2013). Therefore, spawning of the grass puffer is tightly connected with seasonal, lunar and tidal rhythms as well as daily rhythm (Ando et al., 2013).

The grass puffer has a single pair of *kiss2* and *kiss2r*, and the expression levels of *kiss2* and *kiss2r* in the brain and pituitary were augmented during the breeding season in both sexes (Shahjahan et al., 2010b). Their expression levels were decreased by anomalous temperature along with the decrease in *gnrh1*, *fshb* and *lhb* expressions along with gonadal regression (Shahjahan et al., 2017). Moreover, in vivo administration of Kiss2 stimulated *kiss2r* and *gnrh1* expressions in the brain (unpublished data). These results suggest that the Kiss2 system has a stimulatory role in the grass puffer reproduction. Previously, for the neuroanatomical structure of the kisspeptin system in the grass puffer, Ando et al. (2014) showed that *kiss2*-expressing cells were localized in the magnocellular preoptic nucleus (PM) in the POA and *kiss2r*-expressing cells in the POA and the nucleus dorsomedialis thalami (NDM) by ISH. Moreover, the expression levels of both *kiss2* and *kiss2r* showed daily and circadian variations with a peak at Zeitgeber time (ZT) 6 (daytime) and at circadian time 15 (subjective nighttime), respectively (Ando et al., 2014). Here in the present study, the detailed neuroanatomical characterization of the kisspeptin system was intended in the grass puffer brain using IHC method and their daily changes in the number of ir cells were examined to further support the daily regulation of the kisspeptin system.

2.2. Materials and methods

2.2.1. Fish

Wild sexually mature male grass puffer (n = 24) were caught at the spawning ground in Tomioka, Kumamoto and Minamiise, Mie, Japan in June and July. They were transferred to Marine Biological Station, Sado Island Center for Ecological Sustainability, Niigata University, Japan. Fish were reared in indoor tanks (500 L) with flow of seawater under natural photoperiod (LD 14:10). They were fed commercial pellets equivalent to 1% of body weight (BW) daily. The total length of the fish ranged from 9.5 to 17.0 cm, weighing from 15 to 81 g. The gonadosomatic index (GSI, gonadal weight/body weight × 100) ranged from 1.1 to 16.4%. Experimental procedures followed the guidance approved by the Animal Care and Use Committees of Niigata University, Niigata, Japan.

2.2.2. Sample collection and tissue preparation

The fish were anesthetized in 0.01% tricaine methane sulfonate (MS222, Sigma–Aldrich, Tokyo, Japan). Immediately after decapitation, brains were dissected out and were fixed in 4% paraformaldehyde in 0.05 M phosphate buffer (pH 7.3) for two days. For the analysis of daily variation of Kiss2- and Kiss2r-ir cell number, fish were sampled at ZT6 (daytime, n = 6) and ZT21 (nighttime, n = 6). At ZT21, the brains were removed under red dim light. After fixation, the brains were washed in 70% ethanol and preserved at 4 ^{o}C. The brains were dehydrated through a series of ethanol solutions and were embedded in paraplast. The samples embedded in paraffin blocks were sectioned coronally and sagittally into 15 µm thickness. For histological identification of the localization of brain nuclei, one set of sections were stained with toluidine blue. The histological sections were examined under a light microscope and differential brain structures were drawn mentioning different areas using nomenclature from European sea bass brain (Paullada-Salmerón *et al*., 2016b) and tilapia brain (Ogawa *et al*., 2016).

2.2.3. Immunohistochemistry

Generation of antiserum for grass puffer Kiss2 and Kiss2r

Primary antibodies used in this study are listed in Table 2.1. Polyclonal antisera against the grass puffer Kiss2 and Kiss2r were generated in rabbits against synthetic peptides, FIYRRAMKQARTHTRSPVSQEVPT (C-terminal peptide of the grass puffer Kiss2 precursor) and KFVAAEERNNNAVH (C-terminal peptide of the grass puffer Kiss2r), respectively.

Immunohistochemistry procedure

For immunohistochemical localization of the grass puffer Kiss2 and Kiss2r, brains were sectioned (15 µm) and mounted onto 3-aminopropylsilane (APS)-coated glass slides. Immunohistochemical procedure was conducted with a Vectastain ABC Elite kit (Vector Laboratories, Inc., CA) following the manufacturer's instruction with minor modification. In brief, the sections were deparaffinized, rehydrated through graded ethanol, and added methanol and hydrogen peroxide mixer solution for the removal of endogenous peroxidase. Then, the slides were dipped into 0.5% casein-phosphate buffered saline (PBS, 0.01M, pH 7.0). The rehydrated sections were pre-incubated in 1% normal goat serum in PBS and then incubated with specific antibody for Kiss2 (1:3000) or Kiss2r (1:3000) in moist chamber at 4^{o}C for overnight. After washed in PBS, the sections were incubated with biotinylated anti-rabbit IgG (1:200) at room temperature for 30 min. Avidin-Biotin Complex solution was then applied. The sections were visualized after the application of 0.05% 3, 3'-diaminobenzidine with 0.03% H_2O_2 in 0.05 M Tris-HCl (pH 7.5). After dehydration, sections were cleared in xylene, coverslipped with DPX mountant (Fisher Chemical). Section's images were captured using a microscope (OLYMPUS BX53, Japan) combined with a digital camera (OLYMPUS DP73, Japan). Two sets of alternate brain sections were incubated for negative controls, with either anti-grass puffer GnIH or GnIH-R antiserum that had been pre-absorbed with the respective antigens (10 µg/ml) for overnight.

2.2.4. Statistical analysis

The ir cell numbers are expressed as means ± standard error of the mean (SEM). Differences in the cell number of Kiss2- and Kiss2r-ir cells between daytime and nighttime were tested by Student t test. Statistical significance was set at $p < 0.05$.

2.3. Results

2.3.1. Localization of Kiss2- and Kiss2r-ir cells in the brain

The localization of Kiss2- and Kiss2r-ir cells is shown in Fig. 2.1. The IHC technique localized single population of Kiss2-ir cells in the POA, mainly in the magnocellular preoptic nucleus pars magnocellularis (PMm) (Fig. 2.1B). Kiss2-ir cells were round or oval shaped and sized ranging from 7 to 15 μm (Figs. 2.2A and 2.2B). Pre-absorption with the antigen peptide successfully eliminated the staining of the cells (Fig. 2.2C). Kiss2-ir fibers could not be detected in the present study.

Kiss2r-ir cells were localized in three brain regions, in the PMm, nucleus dorsomedialis thalami (NDM) and the medial preglomerular nucleus (Pgm) (Figs. 2.1B, 2.1C and 2.1D). In the PMm, Kiss2r-ir cells were likely to show similar shape and size with Kiss2-ir cells (Figs. 2.3A and 2.3B). In the dorsal thalamus, smaller number of Kiss2r-ir cells were scattered mainly in the NDM (Figs. 2.3C and 2.3D). Third population of Kiss2r-ir cells was observed in the Pgm in the mesencephalon of the grass puffer brain (Figs. 2.3E and 2.3F). Most of the Kiss2r-immunoreactivities were successfully blocked by pre-absorption with the antigen peptide (Figs. 2.3G and Fig. 2.3H).

2.3.2. Daily variation in the number of Kiss2- and Kiss2r-ir cells

To examine daily changes in the number of Kiss2-ir and Kiss2r-ir cells, ir cells in all the sections containing ir signals from six brains at each time (ZT6 or ZT21) were counted in the corresponding brain areas. In the PMm, the number of Kiss2-ir cells was significantly higher at ZT21 during the nighttime compared to ZT6 during the daytime (Fig. 2.4). The number of

Kiss2r-ir cells was also higher during the nighttime than daytime in the PMm and NDM, but no significant difference was observed in the Pgm (Fig. 2.5).

2.4. Discussion

In the present study, to clarify the neuroanatomical organization of the kisspeptin system in the brain of grass puffer, specific antibodies were raised against the grass puffer Kiss2 and Kiss2r. For the Kiss2 IHC, the C-terminal peptide of the grass puffer Kiss2 precursor was used as antigen according to Servili et al. (2011), in which anti-zebrafish Kiss1/2 precursor antisera were successfully used in the IHC study. In general, it is difficult to localize neuropeptide-producing soma using antibody against mature peptide due to intracellular transportation and rapid release from the neurosecretory cells. Therefore, most studies of brain localization of kisspeptin neurons have been done using ISH technique. In the present study, antibody against the grass puffer Kiss2 precursor could be used to detect specific Kiss2 immunoreactivity since the pre-absorption with the antigen peptide eliminated the signals. Likewise, the antibody against the grass puffer Kiss2r was successfully used to localize the ir cells. This is the first report of immunolocalization of kisspeptin receptor-producing neurons in vertebrates.

The present results revealed that Kiss2-ir cells are located in only single cell population in the grass puffer brain, the PMm in the POA. This is consistent with the previous results of Ando et al. (2014) showing the *kiss2*-expressing cells in the same nucleus. Moreover, the localization in the POA of *kiss1*-expressing cells in goldfish (Kanda et al., 2012) and *kiss2*-expressing cells in zebrafish support the present results. Besides, Kiss2-ir cells were mostly found in the dorsal, lateral and ventral hypothalamus in zebrafish, and these neurons send projections widely toward the subpallium, the entopeduncular nucleus, the POA, the thalamus, the ventral and caudal hypothalamus, and the torus semicircularis (TS) (Servili et al. 2011; Song et al., 2015). In masu salmon, *kiss2*-expressing cells were mainly located in the nucleus recessus lateralis (NRL) in the hypothalamus along with a small population in the POA

(Osugi et al., 2013). These differential patterns of distribution could be due to interspecies variation or its low expression levels in the hypothalamus.

The Kiss2r-ir cells displayed a broader distribution in three brain regions, the PMm of the POA, NDM and Pgm. The distribution pattern of *kiss2r* expression was previously determined by Ando et al., (2014), in which *kiss2r*-expressing cells are consistently localized in the PMm and NDM. So far, some differences in the distribution of *kiss2r*-expressing cells have been reported in other fishes. For example, *kiss2r*-expressing cells were detected in the dorsal telencephalon, habenula, hypothalamus and optic tectum in goldfish (Li et al. 2009) and zebrafish (Servili et al. 2011). In medaka, *kiss2r* was mainly expressed in the ventral telencephalon, POA and hypothalamus (Kanda et al. 2013). In chub mackerel, *kiss2r*-expressing cells were detected in the POA and the lateral tuberal nucleus and NRL in the hypothalamus (Ohga et al. 2017). In striped bass, *kiss2r* mRNAs was colocalized in GnRH1 neurons in the POA (Zmora et al. 2012). These different and somewhat scattered localization in the hypothalamus of Kiss2r neurons suggest a multifunctional role of Kiss2 involved in reproduction as well as non-reproduction activities in various fishes.

It should be also noted that both Kiss2-ir and Kiss2r-ir cells were localized in the PMm of the POA. The distribution and morphological character of the Kiss2-ir and Kiss2r-ir cells are quite similar, suggesting that some of the PMm neurons co-express Kiss2 and Kiss2r. Thus, the autocrine action of Kiss2 is most probably feasible in the grass puffer, that has not yet reported before. Furthermore, since the PMm is one of the major hypothalamic nuclei that consists of multiple peptidergic neurons including GnRH and GnIH (Chapter 3), the functional interaction of these neuropeptides is also possible in the PMm to regulate reproduction in the grass puffer.

To confirm the daily regulation of *kiss2* and *kiss2r* expression (Ando et al., 2014), the number of Kiss2- and Kiss2r-ir cells in the brains sampled at daytime and nighttime were counted. Both the number of Kiss2- and Kiss2r-ir cells was significantly higher at nighttime when compared to daytime. Previously, the grass puffer *kiss2* and *kiss2r* mRNA levels showed clear daily variations with one peak at ZT6 (middle of the daytime) (Ando et al.,

2014) that interestingly differ from the present results. In this current study, the immunoreactivities detected are considered to correspond to intracellular amounts of Kiss2 and Kiss2r molecules instead of their mRNA levels and the difference may happen due to post-transcriptional regulation where 10–14 h of time interval needed between transcription and appearance of protein (Ikegami et al., 2009). Taken together, the present results support the time-dependent activity of the kisspeptin system in the brain that may be important for the precisely timed spawning of the grass puffer.

Table 2.1. List of primary antisera used in the present study.

Name of the antibody	**Structure of the immunogen**	**Species in which raised, monoclonal/ polyclonal**	**Dilution for IHC**
Anti-grass puffer Kiss-2 antibody	FIYRRAMKQARTHTRSPVSQE VPT (synthetic C-terminal peptide of the grass puffer Kiss2 precursor) conjugated with keyhole limpet hemocyanine (KLH)	rabbit, polyclonal	1:3000
Anti-grass puffer Kiss2r antibody	KFVAAEERNNNAVH (synthetic C-terminal peptide of the grass puffer Kiss2r) conjugated with keyhole limpet hemocyanine (KLH)	rabbit, polyclonal	1:3000

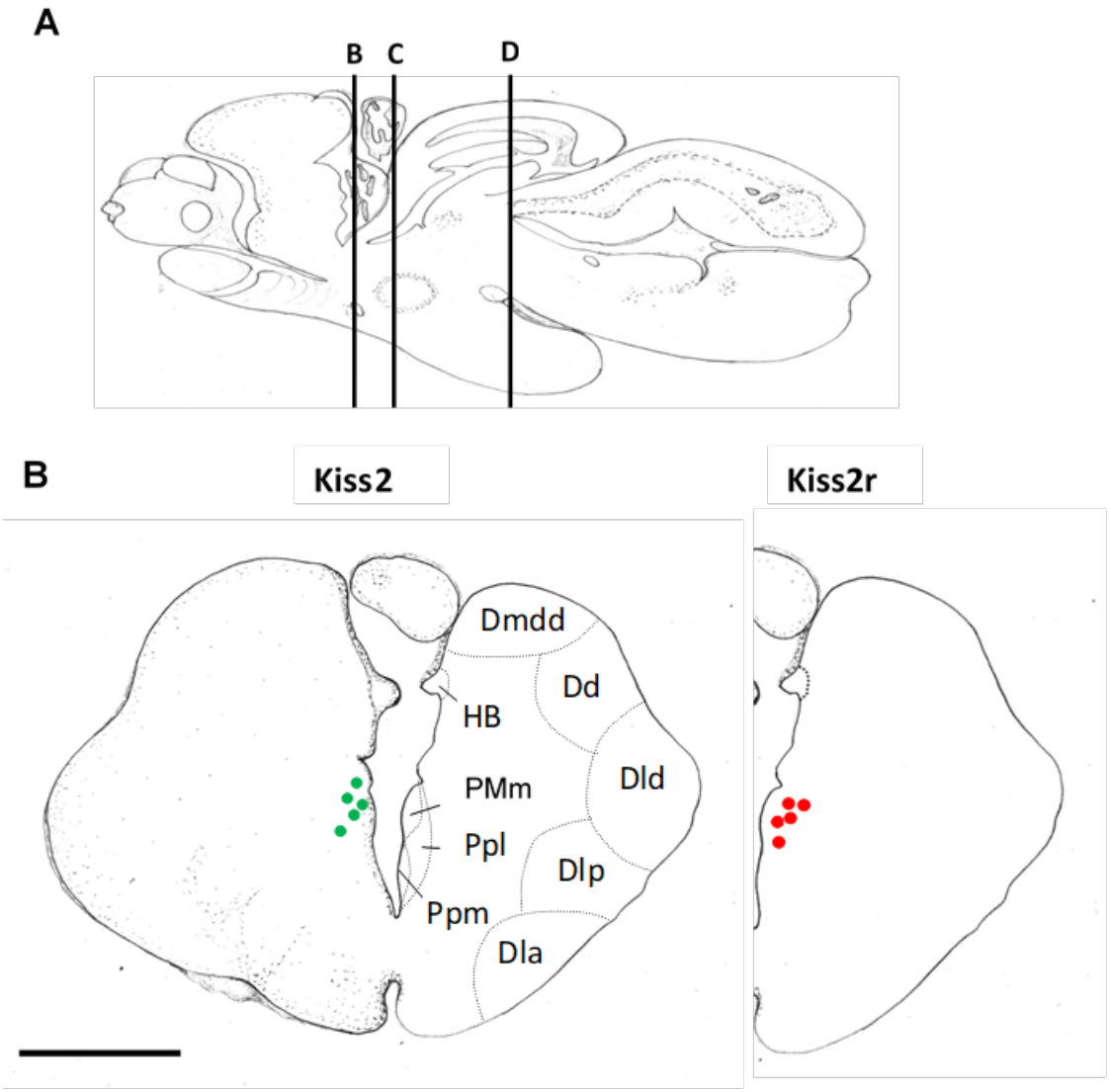


Fig. 2.1. Schematic drawings of coronal sections through the grass puffer brain showing the distribution of Kiss2-ir cells and Kiss2r-ir cells. A: Lines on a schematic sagittal drawing indicate positions of coronal sections (B-D) of the grass puffer brain. B-D: Left column of coronal brain sections shows Kiss2-ir cells (green circles). Right column labeled for Kiss2r-ir cells (red circles). Scale bars = 1 mm. Dl, lateral part of the dorsal telencephalon; NAPv, anterior periventricular nucleus; NDLI, diffuse nucleus of the inferior lobe; NDM, nucleus dorsomedialis thalami; NRL, nucleus recessus lateralis; NVM, nucleus ventromeidalis thalami; PMm, magnocellular preoptic nucleus pars magnocellularis; Ppl, lateral part of the posterior preoptic area; Ppm, medial part of the posterior preoptic area; OT, optic tectum; SV, saccus vasculosus; TLa, nucleus of the lateral torus; TLo, longitudinal torus; TS, Semicircular torus; Pgm, medial preglomerular nucleus.

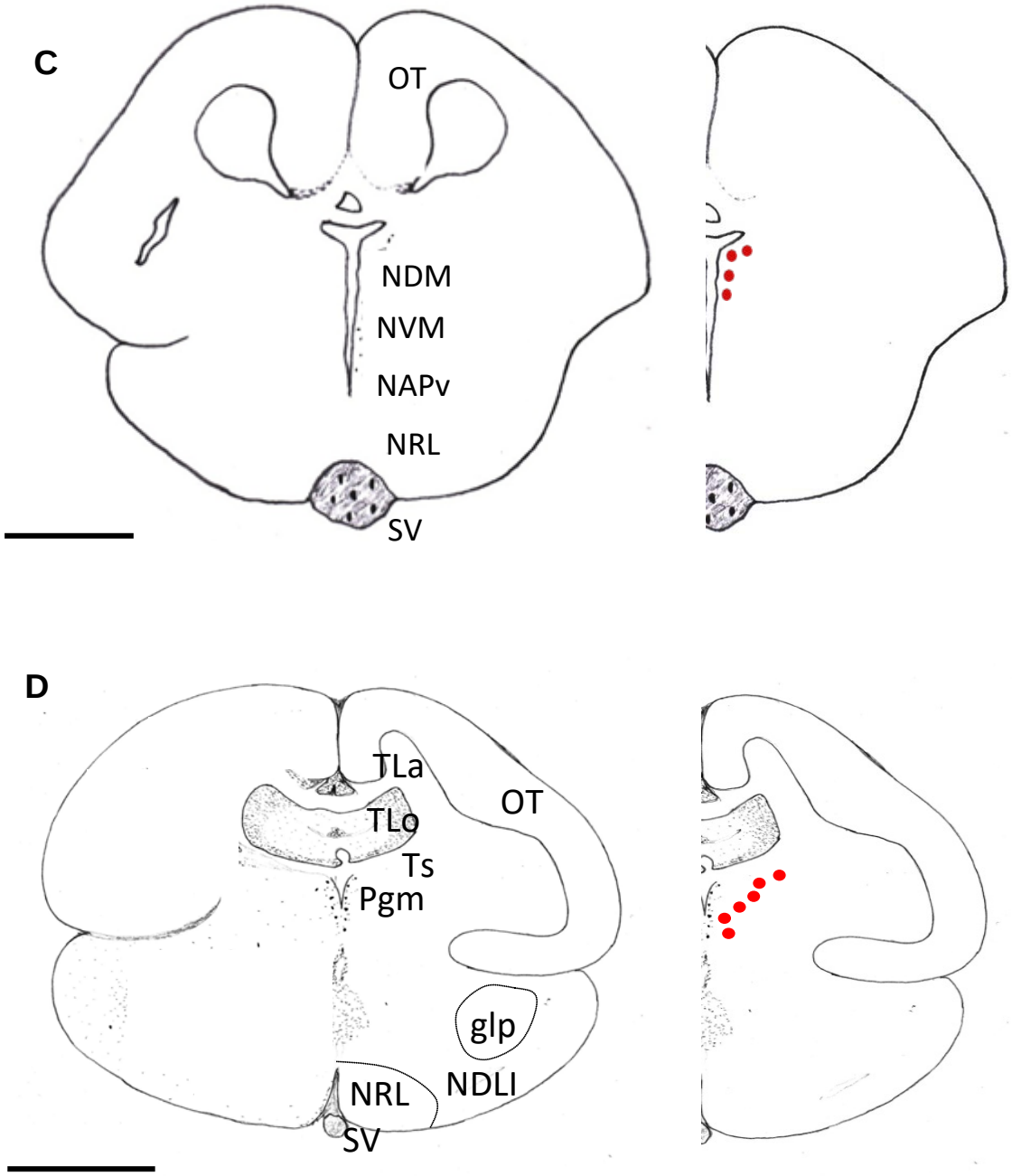


Fig. 2.1. (continued)

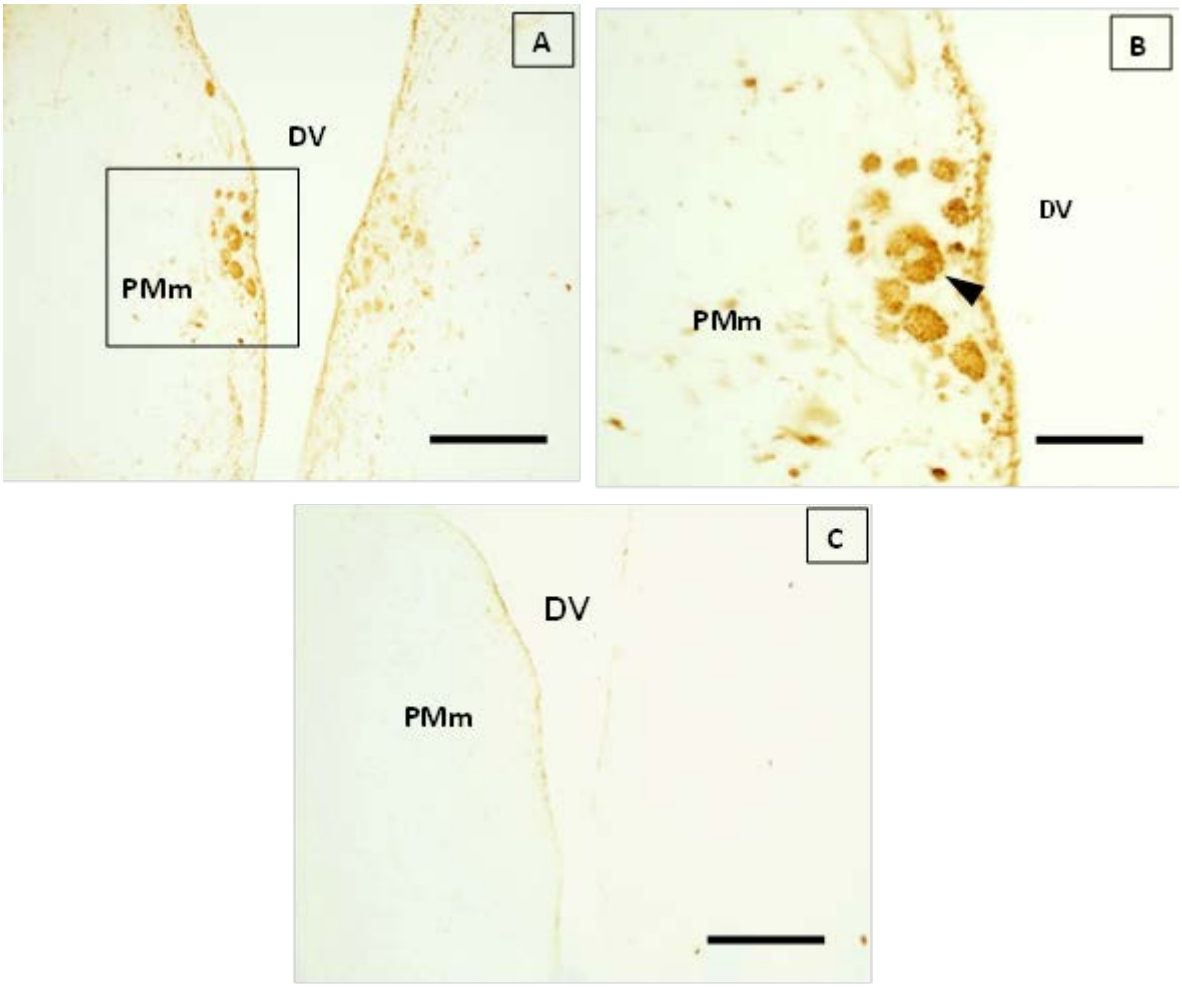


Fig. 2.2. Photomicrographs showing the localization of Kiss2-ir cells in the brain of grass puffer. A-C: Photomicrographs of coronal sections with low (A and C) and high magnification (B) showing Kiss2-ir cells in the magnocellular preoptic nucleus pars magnocellularis (PMm). C: Pre-absorption with the antigen peptide (10 µg/µl) successfully eliminated the Kiss2 immunoreactivity. DV, diencephalic ventricle. Arrow heads indicate Kiss2-ir cells and boxes direct higher magnification sites. Scale bars = 50 µm in A and C; 20 µm in B.

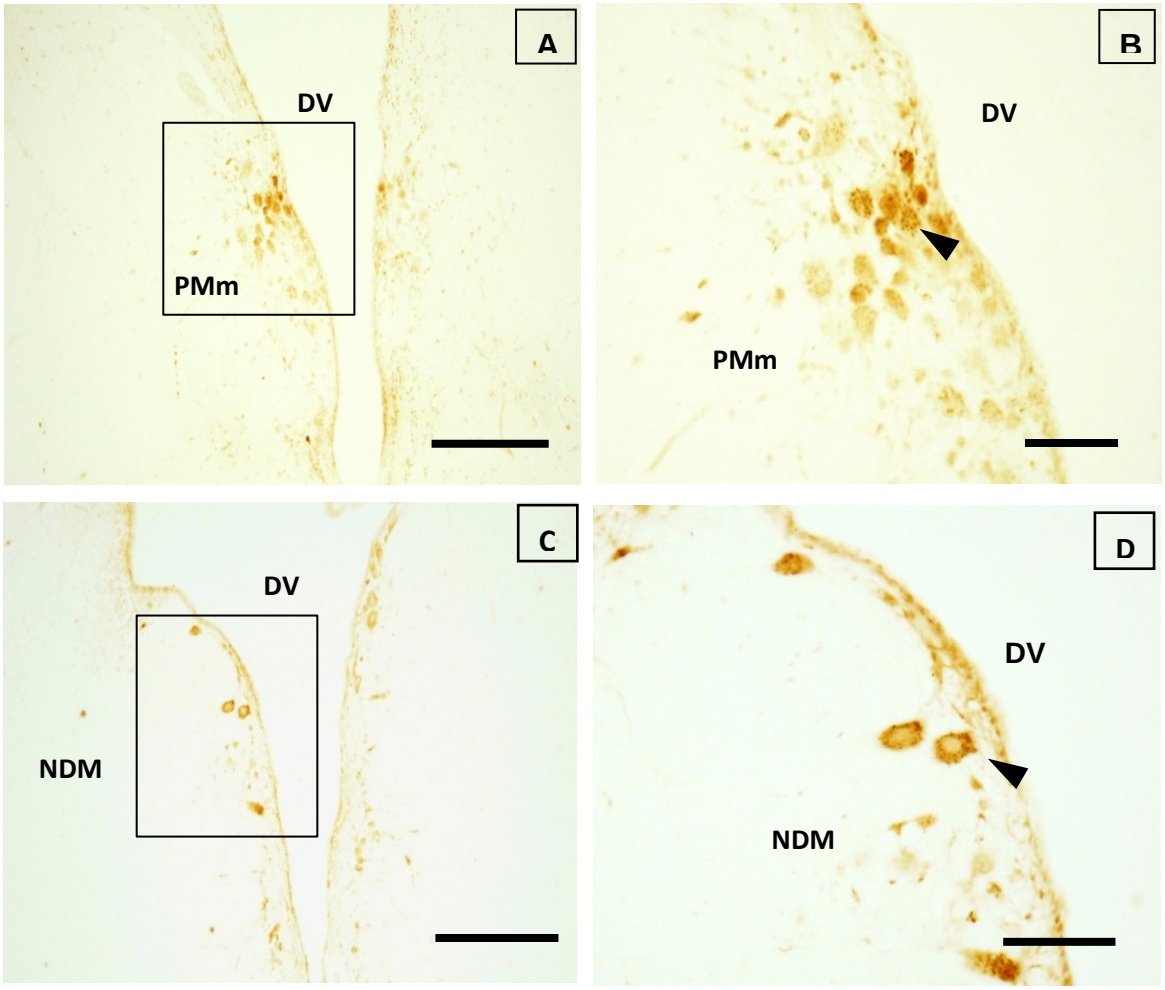


Fig. 2.3. Photomicrographs showing the localization of Kiss2r-ir cells in the brain of grass puffer. A-H: Photomicrographs of coronal sections showing Kiss2r-ir cells in the brain with low (A, C, E, G and H) and high (B, D and F) magnifications. Pre-absorption of anti-Kiss2r antiserum with the antigen peptide (10 µg/µl) eliminated the signals (G and H). A and B: Kiss2r-ir cells in the magnocellular preoptic nucleus pars magnocellularis (PMm). C and D: the nucleus dorsomedialis thalami (NDM). E and F: the medial preglomerular nucleus (Pgm). Arrow heads indicate Kiss2r-ir cells and boxes direct higher magnification sites. Scale bars = 50 µm in A, C, E, G and H; 20 µm in B, D and F.

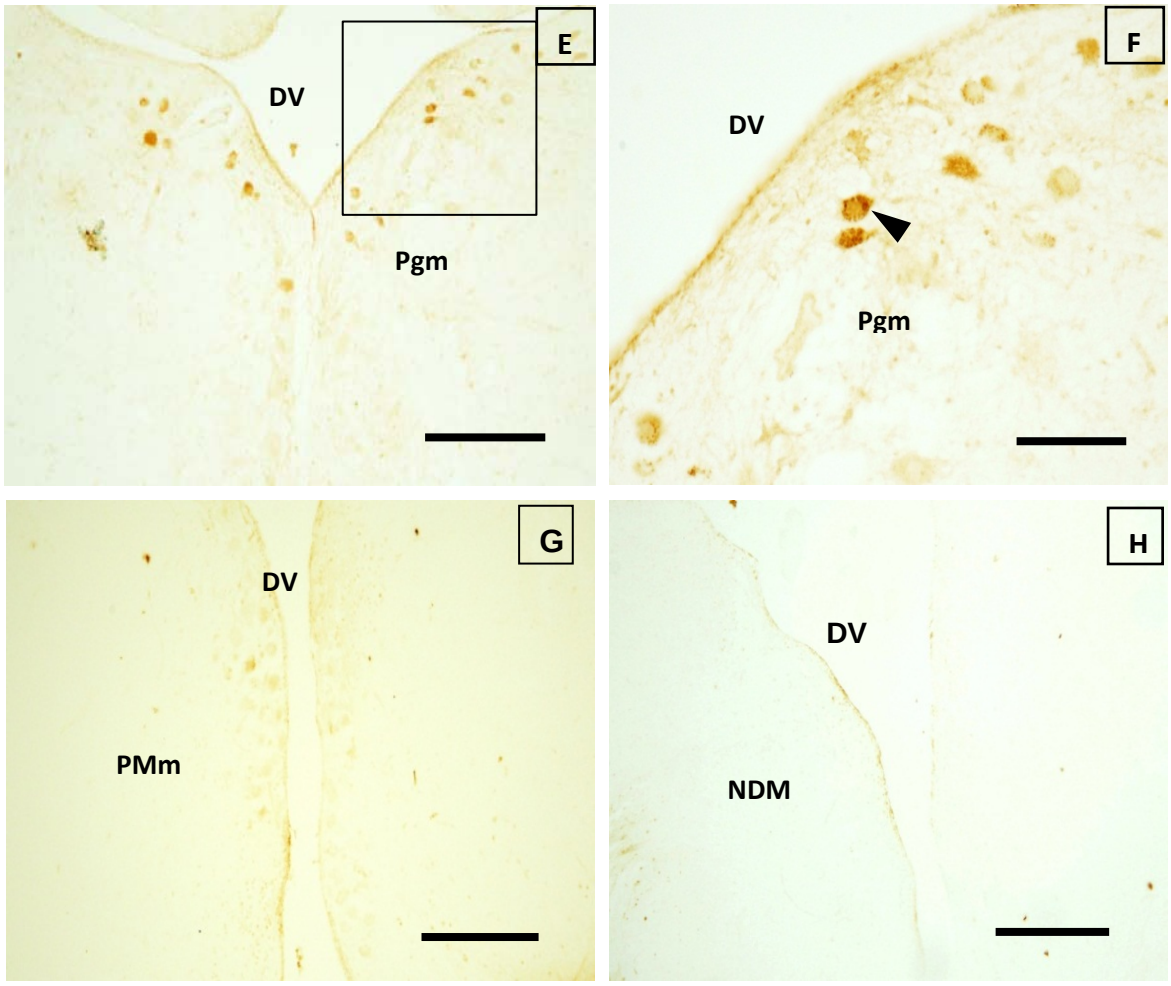


Fig. 2.3. (continued)

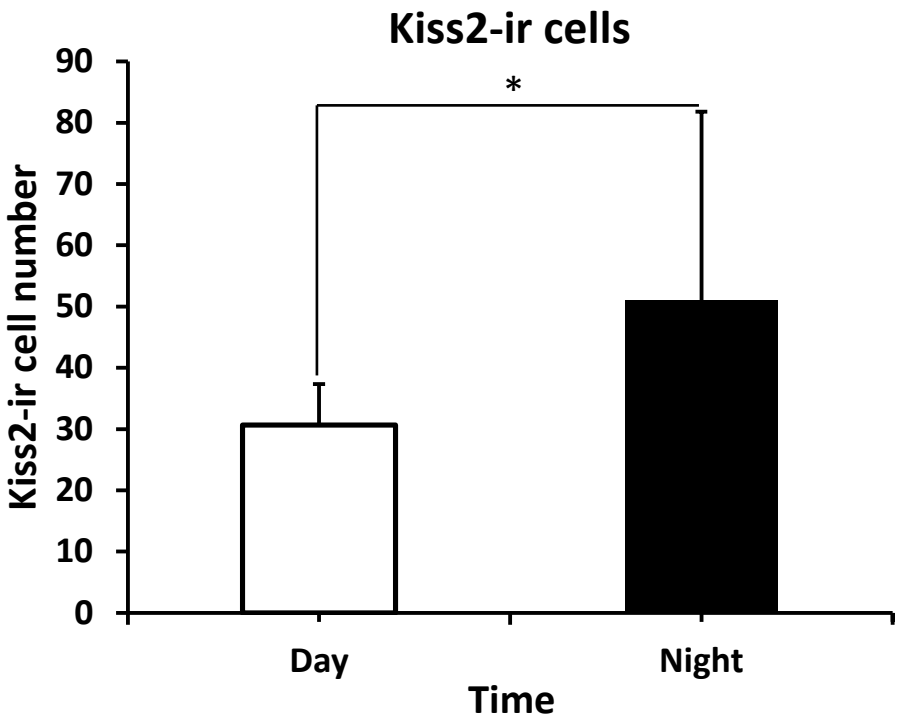


Fig. 2.4. Daily variation in the number of Kiss2-ir cells in the magnocellular preoptic nucleus pars magnocellularis of the grass puffer brain. Values represent the mean ± SEM (n = 6). * $p < 0.05$.

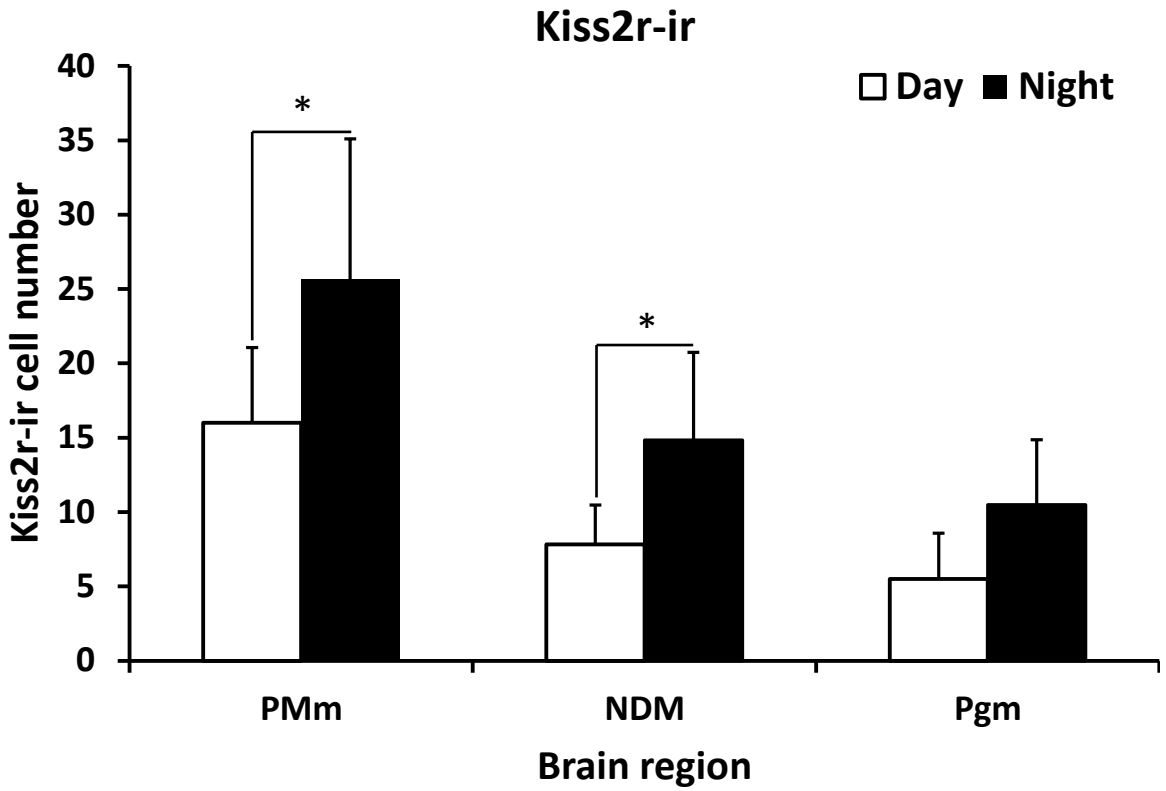


Fig. 2.5. Daily variation in the number of Kiss2r-ir cells in three brain regions of the grass puffer brain. Values represent the mean ± SEM (n = 6). * $p < 0.05$. PMm, the magnocellular preoptic nucleus pars magnocellularis; NDM, the nucleus dorsomedialis thalami; Pgm, the medial preglomerular nucleus.

Chapter 3

Neuroanatomical studies on the GnIH and GnIH receptor system

3.1. Introduction

Reproduction in vertebrates is regulated by the hypothalamic–pituitary–gonad (HPG) axis where gonadotropin-releasing hormone (GnRH) serves as a stimulatory hypothalamic neuropeptide in many vertebrates, but no inhibitory hypothalamic factors of the HPG axis had been unknown for a long time. From the discovery of gonadotropin-inhibitory hormone (GnIH) in 2000, first identified in the Japanese quail (*Coturnix japonica*) as a suppressor of LH release (Tsutsui et al., 2000), studies on orthologs of this new hypothalamic factor has gained much attention in many vertebrate species. It is now acknowledged generally that GnIH plays as an important hypophysiotropic neurohormone together with GnRH for it differentially regulates the secretion of gonadotropins (GTHs), growth hormone (GH) and prolactin (PRL) from the pituitary (Ogawa and Parhar, 2014; Ubuka et al., 2016). In fish, GnIH actions seem more complex showing dual roles depending on species and gonadal stage. GnIH has been shown to stimulate or inhibit synthesis and release of GTHs and GH in a variety of teleost species (Amano et al., 2006; Zhang et al., 2010; Shahjahan et al., 2011; Moussavi et al., 2012, 2014; Qi et al., 2013; Biran et al., 2014; Wang et al., 2015; Choi et al., 2016; Di Yorio et al., 2016; Peng et al., 2016; Paullada-Salmerón et al., 2016a). To clarify the role of GnIH on the regulation of fish reproduction, the anatomical localization of brain cells producing GnIH and GnIH receptor (GnIH-R) is prerequisite, giving fundamental and useful information to consolidate the complex actions of GnIH.

The presence and distribution of GnIH and GnIH-R in the brain has been examined in various vertebrate species by using various methods including immunoassays, PCR,

immunohistochemistry (IHC) and in situ hybridization (ISH). In mammals, GnIH-immunoreactive (ir) cells has been identified mainly in the hypothalamus such as in the paraventricular nucleus, dorsomedial nucleus, mediobasal and ventromedial hypothalamus, as well as in the olfactory bulbs, hippocampus, medulla oblongata and spinal cord. In bullfrog, frog GH-releasing peptide (fGRP), a GnIH ortholog, ir cell bodies were identified in the medial septal area, preoptic area (POA) and suprachiasmatic nucleus and they innervated the median eminence (Koda et al., 2002). Above all, the arrangement of GnIH innervation in the brain of tetrapods is consistent with the presence of GnIH-R (Ubuka et al., 2013).

In fish, however, limited studies have been made on the localization of GnIH neurons due to the lack of specific antibodies against fish GnIH orthologs. So far, GnIH-ir cells have been shown to be localized in the posterior periventricular nucleus (NPPv) of the caudal POA of goldfish, sockeye salmon, Indian major carp, sea bass and tilapia (Sawada et al., 2002: Amano et al., 2006; Biswas et al., 2015; Paullada-Salmerón et al., 2016b; Di Yorio et al., 2016; Ogawa et al., 2016), as well as in the magnocellular preoptic nucleus (PM) and periventricular preoptic nucleus of Indian major carp (Biswas et al., 2015). Moreover, GnIH-ir perikarya were found in the dorsal mesencephalic tegmentum and in the rhombencephalon (Biswas et al., 2015; Di Yorio et al., 2016).

GnIH-R, a G-protein-coupled receptor (GPR), GPR147, was first identified in birds (Ikemoto and Park, 2005; Yin et al., 2005) and their cognate receptors have been also identified in zebrafish (Zhang et al., 2010), grass puffer (Shahjahan et al., 2011), goldfish (Qi et al., 2013) and tilapia (Biran et al., 2014). Although the expression of GnIH-R gene has been determined by real-time PCR analysis (Zhang et al., 2010; Shahjahan et al., 2011), the detailed neuroanatomical localization of GnIH-R has been limited in fish due to the lack of specific antibodies against fish GnIH-R. In tilapia, GnIH-R-ir cells were found to be widely distributed throughout the brain, particularly in the POA, hypothalamus, optic tectum, semicircular torus (TS), and caudal midbrain tegmentum (Ogawa et al., 2016). The ISH study

on the GnIH-R gene also showed similar results in goldfish, in which three subtypes of GnIH-R genes were expressed in the POA, NPPv and lateral tuberal nucleus and two GnIH-R subtype genes were expressed in the pars intermedia of the pituitary (Qi et al., 2013).

The grass puffer (*Takifugu alboplumbeus*) is an intertidal puffer species, having an interesting and unique spawning behavior and its spawning is synchronized with lunar and tidal cycles. In early summer from May to July, they aggregate at certain seashore place for spawning 2–3 h before high tide at dusk only during spring tide every two weeks, and then spawning occurs 1–2 h before high tide during flood tide (Motohashi et al., 2010; Ando et al., 2013). Therefore, spawning of the grass puffer is tightly connected with seasonal, lunar and tidal rhythms as well as daily rhythm (Ando et al., 2013). The grass puffer provides an excellent animal model for studying the molecular and neuroendocrine mechanisms of the periodic regulation of reproduction.

The grass puffer GnIH system is composed of a precursor of 173 amino acid (aa) residues that is cleaved into three putative peptides, GnIH-1, GnIH-2 and a possible RYamide peptide, and GnIH-R of 474 aa residues (Shahjahan et al., 2011). The expression levels of *gnih* and *gnih-r* were significantly augmented in the brain and pituitary during the spawning period in both sexes, and both genes showed daily and circadian oscillations in expression in the brain (Shahjahan et al., 2011). Moreover, the goldfish LPXRFa peptide-1 stimulated the expression of *fshb* and *lhb* (Shahjahan et al., 2011) and *gh* and *prl* (Shahjahan et al., 2016) in the cultured pituitary cells of the grass puffer. These results suggest that GnIH may act as a key player regulating reproduction through stimulating the secretion of GTHs and GH in the grass puffer. Therefore, in the present study, the brain localization of GnIH and GnIH-R were examined in the grass puffer brain to further establish the regulatory role of the GnIH/GnIH-R system.

3.2. Materials and methods

3.2.1. Fish

Wild sexually mature male grass puffer (n = 8) was collected at the spawning ground in Miyazaki, Japan in May. The total length of the fish ranged from 12.7 to 15.0 cm, weighing from 44 to 64 g. The gonadosomatic index (GSI, gonadal weight/body weight × 100) ranged from 12 to 17%. Experimental procedures followed the guidance approved by the Animal Care and Use Committees of Niigata University, Niigata, Japan.

3.2.2. Tissue preparation

The fish were anesthetized in 0.01% tricaine methane sulfonate (MS222, Sigma–Aldrich, Tokyo, Japan). Immediately after decapitation, brains were dissected out and were fixed in 4% paraformaldehyde in 0.05 M phosphate buffer (pH 7.3) for 3 days. After fixation, the brains were washed in 70% ethanol and preserved at 4 ^{o}C for further histological analysis. The brains were dehydrated through a series of ethanol solutions and were embedded in paraplast. The samples embedded in paraffin blocks were sectioned coronally and sagittally into 15 µm thickness. For histological identification of the localization of brain nuclei, one set of sections were stained with toluidine blue. The histological sections were examined under a light microscope and differential brain structures were drawn mentioning different areas using nomenclature from European sea bass brain (Paullada-Salmerón et al., 2016b) and tilapia brain (Ogawa et al., 2016).

3.2.3. Immunohistochemistry

Generation of antiserum for grass puffer GnIH and GnIH-R

Primary antibodies used in this study are listed in Table 3.1. Polyclonal antisera against the grass puffer GnIH and GnIH-R were generated in rabbits against synthetic peptides,

DGVQGGDHVPNLNPNMPQRFamide (the grass puffer GnIH-2) and VSSLEASVYKAWEK (C-terminal peptide of the grass puffer GnIH-R), respectively.

Immunohistochemistry procedure

For immunohistochemical localization of the grass puffer GnIH and GnIH-R, brains (n = 6) were sectioned (15 µm) and mounted onto 3-aminopropylsilane (APS)-coated glass slides. Immunohistochemical procedure was conducted with a Vectastain ABC Elite kit (Vector Laboratories, Inc., CA) following the manufacturer's instruction with minor modification. In brief, the sections were deparaffinized, rehydrated through graded ethanol, and added methanol and hydrogen peroxide mixer solution for the removal of endogenous peroxidase. Then, the slides were dipped into 0.5% casein-phosphate buffered saline (PBS, 0.01M, pH 7.0). The rehydrated sections were pre-incubated in 1% normal goat serum in PBS and then incubated with specific antibody for GnIH (1:500) or GnIH-R (1:500) in moist chamber at 4^{o}C for overnight. After washed in PBS, the sections were incubated with biotinylated anti-rabbit IgG (1:200) at room temperature for 30 min. Avidin-Biotin Complex solution was then applied. The sections were visualized after the application of 0.05% 3, 3'-diaminobenzidine with 0.03% H_2O_2 in 0.05 M Tris-HCl (pH 7.5). After dehydration, sections were cleared in xylene, coverslipped with DPX mountant (Fisher Chemical). Section's images were captured using a microscope (OLYMPUS BX53, Japan) combined with a digital camera (OLYMPUS DP73, Japan). Two sets of alternate brain sections were incubated for negative controls, with either anti-grass puffer GnIH or GnIH-R antiserum that had been pre-absorbed with the respective antigens (10 µg/ml) for overnight.

3.3. Results

3.3.1. Localization of GnIH-ir cells in the brain

The distribution of GnIH-ir cells and neuronal processes is shown in Fig. 3.1 and relative densities of the immunoreactivities of GnIH-ir cells and fibers are summarized in Table. 3.2. GnIH-ir cells were localized in the magnocellular preoptic nucleus pars magnocelluaris (PMm) and TS, which appeared as round or oval shape (Figs. 3.1D, 3.1H and 3.2A-D). Pre-absorption with the antigen peptide successfully blocked the staining of the cells and fibers (Figs. 3.2E and 3.2F). The GnIH-ir fibers were widely distributed in the forebrain, midbrain and hindbrain (Table 3.2, Figs. 3.1 and 3.3). In the telencephalon, GnIH-ir fibers were distributed in the lateral part of the dorsal telencephalon (Dl) (Figs. 3.1C, 3.1D and 3.3A-D). In particular, dense fibers were visible in the dorsal and posterior subdivisions of lateral part of the Dl and anterior subdivision of the medial part of the dorsal telencephalon. In the diencephalon, GnIH-ir fibers were present in the PMm (Fig. 3.1D), nucleus ventromedialis thalami (Fig. 3.1F), anterior periventricular nucleus (Figs. 3.1F, 3.3E and 3.3F) and nucleus of lateral recess (Figs. 3.1F-H). In the mesencephalon, GnIH-ir fibers project in the central zone of the optic tectum (Figs. 3.1H, 3.1I, 3.3G and 3.3H), medial preglomerular nucleus (Pgm) (Fig. 3.1H) and posterior tubercle (Fig. 3.1H and 3.3G-I). In the rhombencephalon, few GnIH-ir fibers were observed in the cerebellum.

3.3.2. Localization of GnIH-R-ir cells in the brain

The distribution of GnIH-R-ir cells is shown in Fig. 3.1 and relative densities of the immunoreactivities of GnIH-R-ir cells are summarized in Table. 3.2. GnIH-R-ir cells were localized mainly in three brain regions. In the telencephalon, GnIH-R-ir cells were localized in the ventral part of the ventral telencephalon (Vv) and medial subdivision of central part of the dorsal telencephalon (Dcm) (Figs. 3.1C and 3.4A-C). In the diencephalon, GnIH-R-ir cells were shown in the PMm and anteroventral part of the magnocellular preoptic nucleus pars parvocellularis (PMp) (Figs. 3.1D, 3.4D and 3.4E). In the diencephalic regions, GnIH-R-ir cells were round shape and larger than the telencephalic region. In the mesencephalon,

majority of the cells were observed in the Pgm (Figs. 3.1H, 3.1I, 3.4F and 3.4G). Pre-absorption with the antigen peptide successfully blocked the GnIH-R immunoreactivity (Figs. 3.4H and 3.4I).

3.4. Discussion

In the brain of grass puffer, localization of GnIH-ir cells was examined applying IHC method with antiserum against the homologous grass puffer GnIH-2. Foremost population of GnIH-ir cells was located in the PMm, in agreement with the result in Indian major carp (Biswas et al., 2014). Besides, the abundance of GnIH-ir soma in the NPPv of the caudal preoptic area has been reported in many in teleost species (Sawada et al., 2002: Amano et al., 2006; Biswas et al., 2015; Paullada-Salmerón et al., 2016b; Di Yorio et al., 2016; Ogawa et al., 2016). The PMm and NPPv are considered to be similar or corresponding neural populations in teleost fishes, since the anatomical information of fish brain has not yet been established due to high variation of neural structures in a variety of fish species. Therefore, the present study suggests that in the grass puffer brain GnIH cell soma are localized in the PMm, which is a common teleost GnIH cell population and one of major nuclei of hypophysiotropic neurosecretory cells.

Another GnIH-ir cell population was found in the TS in the mesencephalic area in consistent with the result in sea bass (Paullada-Salmerón et al., 2016b). In fish, the TS has been related with the processing of mechanosensorial and auditory stimuli, color vision, prey localization, navigation and schooling behaviors (Schellart, 1983; Etcheler, 1984; Coombs et al., 1989). Interestingly, the cells of the TS represent a target for melatonin, the pineal hormone, in sea bass (Herrera-Perez et al., 2010).

In the present study, widespread innervation of GnIH-ir cells was determined that covers forebrain and midbrain regions including the telencephalon, diencephalon and mesencephalon,

but minor projections reached the hindbrain and olfactory bulb. A similar GnIH innervation pattern was found in goldfish, sockeye salmon, European sea bass and Indian major carp (Amano et al., 2006; Biswas et al., 2015; Di Yorio et al., 2016; Paullada-Salmerón et al., 2016b; Sawada et al., 2002). The wide distribution of GnIH-ir fibers suggests a variety of brain functions of GnIH such as neuromodulatory as well as neuroendocrine factors. Profound GnIH-ir fibers have been found in the POA where GnRH cell populations are located (Pandolfi et al., 2002; Stefano et al., 2000). Recently, in tilapia, has revealed the lack of the relationship of GnIH with GnRH showing no close association between GnIH- and GnRH1- or GnRH3-ir cells or fibers (Ogawa et al., 2016; Di Yorio et al., 2019), but colocalization of GnIH and GnRH3 was found in the nucleus olfacto-retinalis in South American cichlid fish (Di Yorio et al., 2019). Moreover, GnIH-ir fibers co-localized with GnRH3-ir cells in the zebrafish forebrain region (Spicer et al., 2017). It is, therefore, probable that GnIH-ir cells may have cell-cell or fiber-fiber contact with GnRH neurons to regulate GTH secretion, but this remains to be clarified in the grass puffer.

Neuroanatomical identification of GnIH-R-ir cells in the brain is important to determine the neural targets of GnIH neurons and their potential actions in the HPG axis. In the present study, GnIH-R-ir cells were found in the Vv and Dcm in the telencephalon, the PMm and PMp in the diencephalon and the Pgm in the mesencephalon. These areas receive GnIH-ir fibers (Table 3.2) and are possible direct-action sites. Interestingly, colocalization of GnIH- and GnIH-R-ir cells was found in the PMm, which is one of the most important hypophysiotropic nuclei with localization of other neuropeptides that regulates the anterior pituitary, suggesting autocrine, paracrine and neuronal interactions of GnIH and other neuropeptide neurons in the PMm for the regulation of reproduction. Thus, the present results demonstrate the neuroanatomical basis of the regulatory role of the GnIH/GnIH-R system in the grass puffer.

In addition, GnIH-R-ir cells have been shown to be widely distributed throughout the brain, particularly in tilapia, GnIH-R-ir cells were found in the POA, hypothalamus, optic tectum, TS and caudal midbrain tegmentum. GnIH-R-ir cells in these nuclei and those in the telencephalon and mesencephalon of the grass puffer brain may denote the extra-reproductive functions of the GnIH/GnIH-R system.

Table 3.1. List of primary antisera used in the present study.

Name of the antibody	**Structure of the immunogen**	**Species in which raised, monoclonal/ polyclonal**	**Dilution for IHC**
Anti-grass puffer GnIH antibody	DGVQGGDHVPNLNPNMPQRF amide (synthetic grass puffer GnIH-2) conjugated with keyhole limpet hemocyanine (KLH)	rabbit, polyclonal	1:500
Anti-grass puffer GnIH-R antibody	VSSLEASVYKAWEK (synthetic C-terminal peptide of the grass puffer GnIH-R) conjugated with keyhole limpet hemocyanine (KLH)	rabbit, polyclonal	1:500

Table 3.2. Abundance or sparsity of GnIH-ir cells and fibers and of GnIH-R-ir cells in the brain areas.

		Densities of immunoreactivities[1]		
Abbre-viations	**Name of brain area**	**GnIH cells**	**GnIH fibers**	**GnIH-R cells**
CZ	Central zone of the optic tectum		+++	
D	Dorsal telencephalon		+	
Da	Anterior part of the dorsal telencephalon		++	
Dcm	Medial subdivision of Dc		+++	++
Dd	Dorsal part of the dorsal telencephalon		++	
Dl	Lateral part of the dorsal telencephalon		+++	+
Dla	Anterior subdivision of Dl		++	
Dld	Dorsal subdivision of Dl		+++	+
Dlp	Posterior subdivision of Dl		+++	+
Dlv	Ventral subdivision of Dl		++	+
Dm	Medial part of the dorsal telencephalon			
Dma	Anterior subdivision of Dm		+++	
Dmd	Dorsal subdivision of Dm		+	
Dmdd	Dorsal part of Dmd		+	
DV	Diencephalic ventricle			
DWZ	Deep white zone of the optic tectum			
GC	Central gray		+++	
glp	Posterior part of the glomerular nucleus			

HB	Habenula			
LSo	Lateral septal organ			
MaOT	Marginal optic tract			
NDLIm	Medial part of the diffuse nucleus of the inferior lobe			
NDLI	Diffuse nucleus of the inferior lobe			
NDLIl	Lateral part of the diffuse nucleus of the inferior lobe			
NDLIld	Dorsal subdivision of NDLIl			
NDLIlv	Ventral subdivision of NDLIl			
NAPv	Anterior periventricular nucleus		++	
NDM	Nucleus dorsomedialis thalami		+++	
NVM	Nucleus ventromedialis thalami		+++	
NPPv	Posterior periventricular nucleus of the caudal preoptic area		++	
NPPvl	Lateral part of NPPv		+	
NRL	Nucleus of the lateral recess		++	
nIII	Oculomotor nucleus			
OT	Optic tectum		+++	
Pgm	Medial preglomerular nucleus		+++	+++
PMm	Magnocellular preoptic nucleus pars magnocellualris	+++	++	+++
PMp	Magnocellular preoptic nucleus pars parvocellualris	++	+	++
Ppl	Lateral part of the posterior preoptic area		++	

Ppm	Medial part of the posterior preoptic area		++	
PPv	Ventral periventricular pretectal nucleus		++	
SV	Vascular sac			
SWGZ	Superficial white and gray zone of the optic tectum			
TLa	Nucleus of the lateral torus			
TLo	Longitudinal torus			+
TP	Posterior tubercle		++	+
TS	Semicircular torus	+++		
VCe	Valvula of the cerebellum			
Vd	Dorsal part of the ventral telencephalon		++	+
Vp	Postcommissural nucleus of the ventral telencephalon		+	
Vv	Ventral part of the ventral telencephalon		++	+++

[1] +++, abundant; ++, moderate; +, sparse.

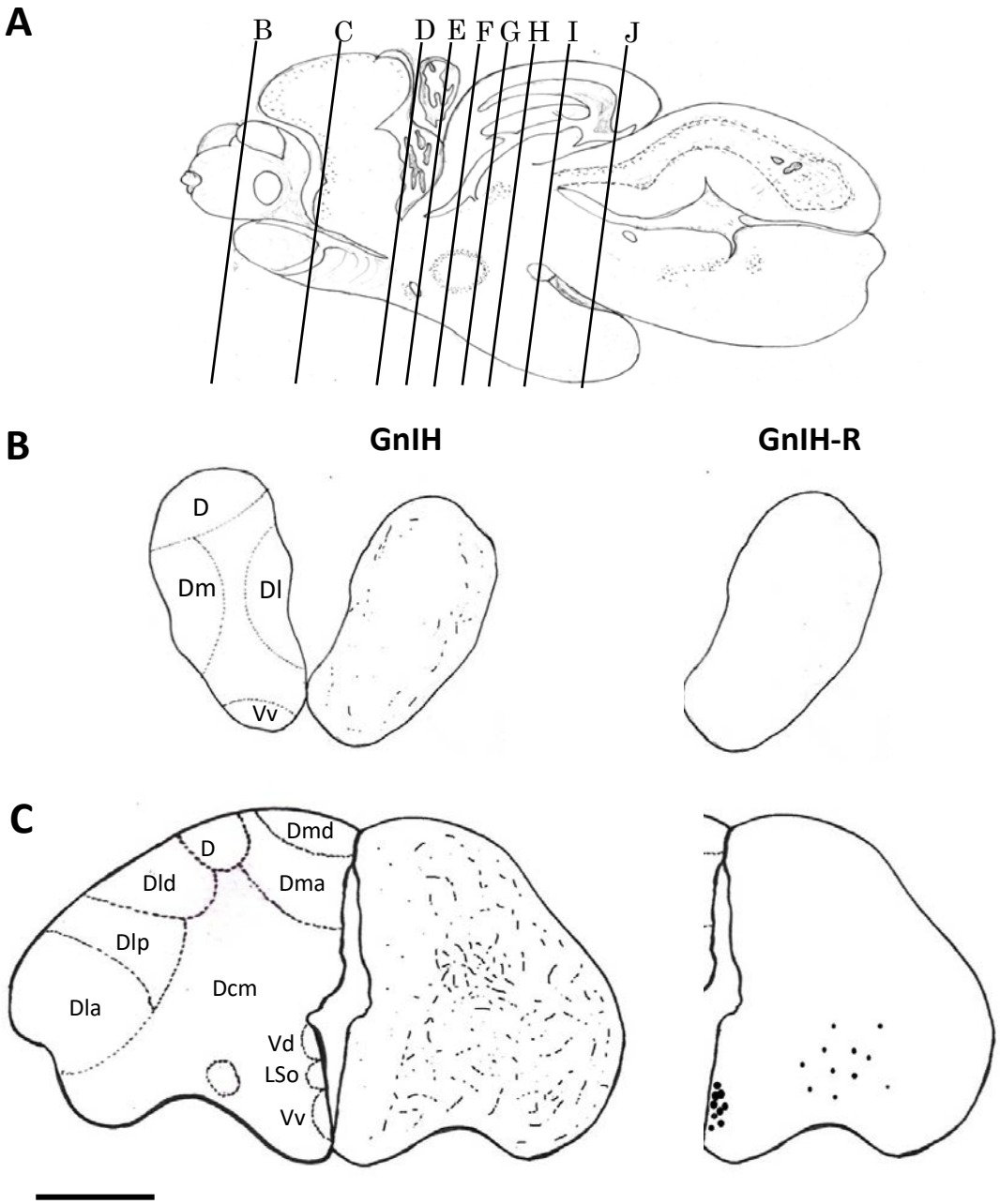


Fig. 3.1. Schematic drawings of coronal sections through the grass puffer brain showing the distribution of GnIH-ir cells and neuronal processes and GnIH-R-ir cells. A: Lines on a schematic sagittal drawing indicate positions of coronal sections (B-J). B-J: Center column of coronal brain sections shows the GnIH-ir cells (black circles) and fibers (small dotted lines). Right column labeled for grass puffer GnIH-R-ir cells (black dots). Abbreviations of the structures in the left column are given in Table 3.2. Scale bars = 1 mm.

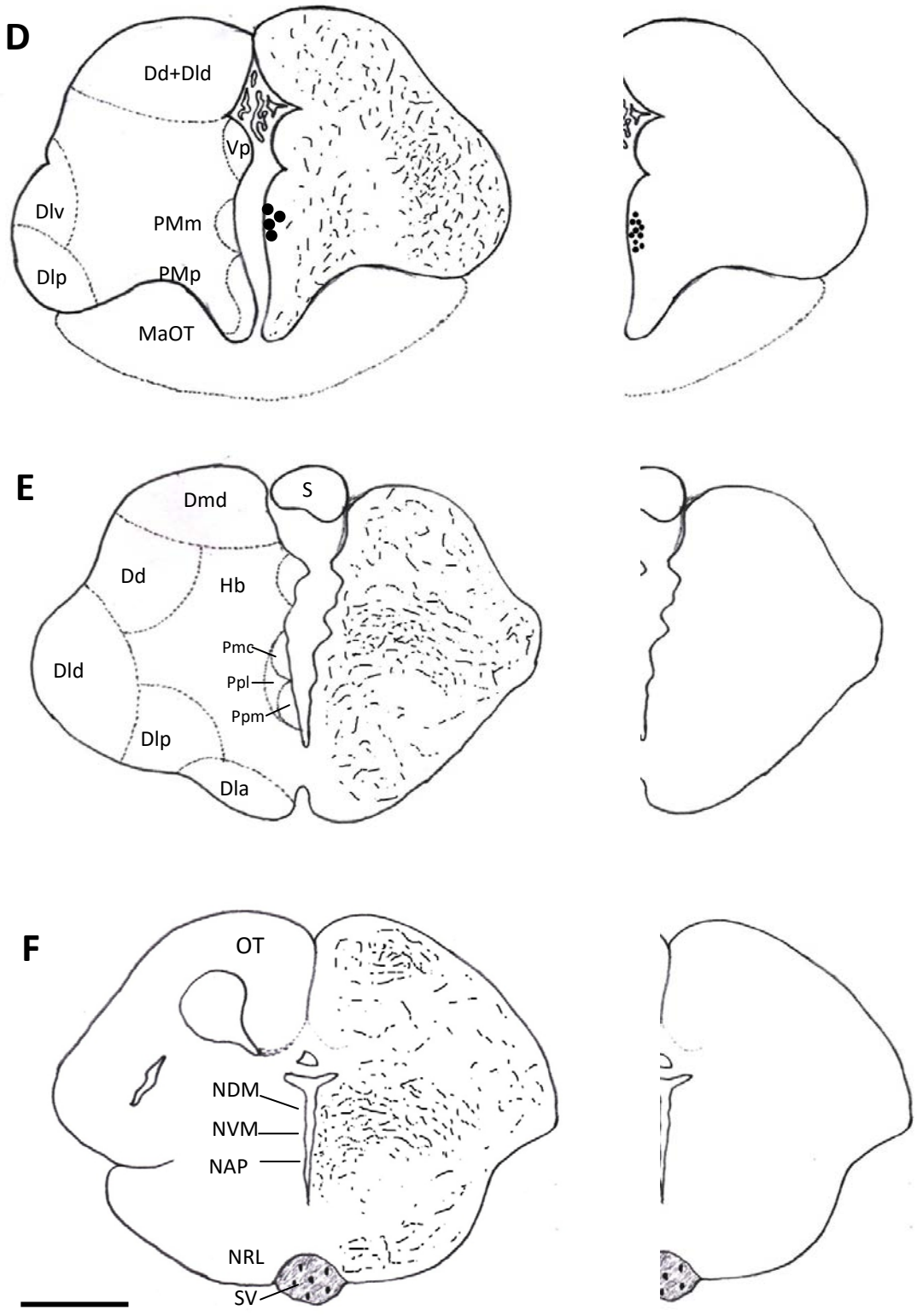
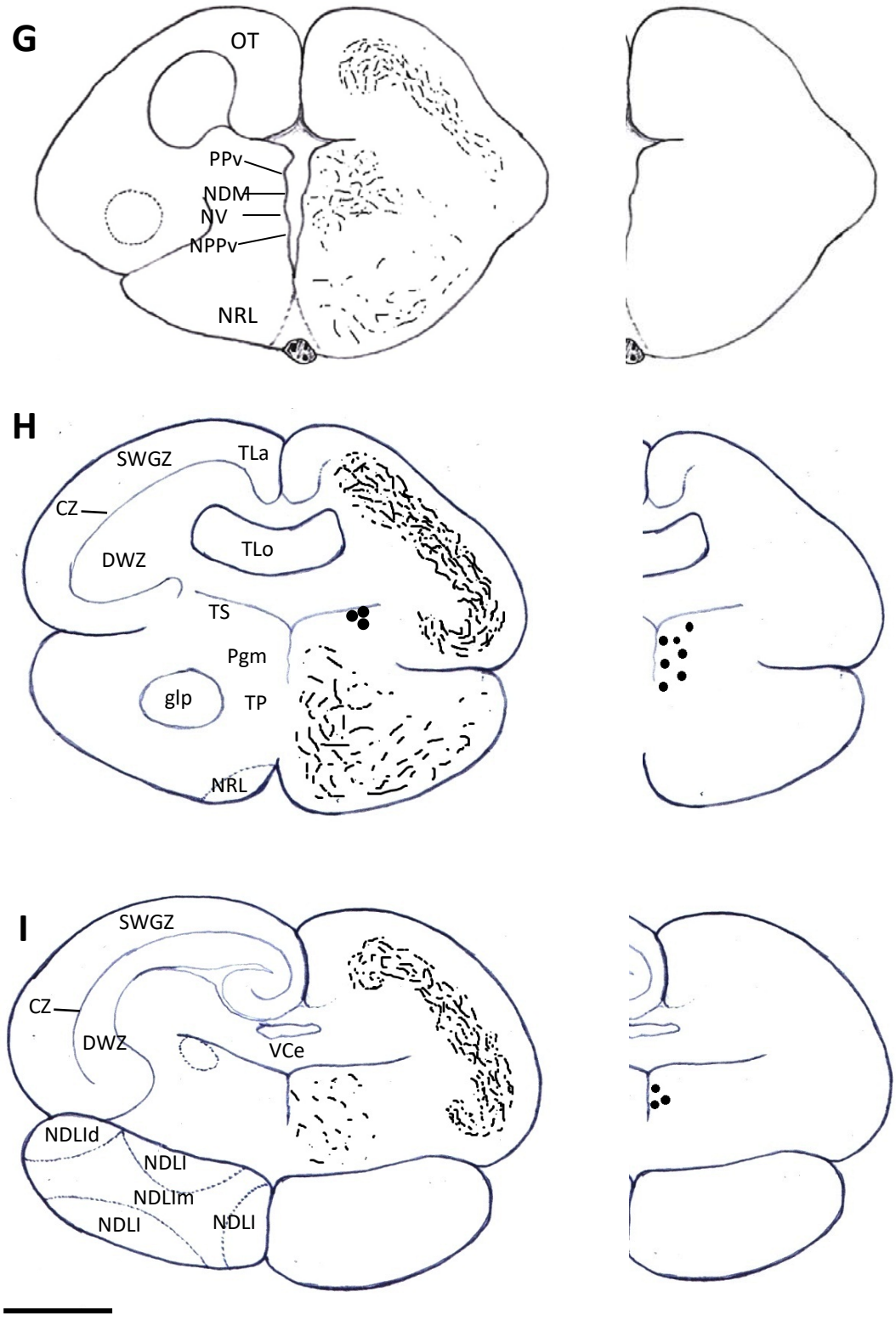


Fig. 3.1. (continued).



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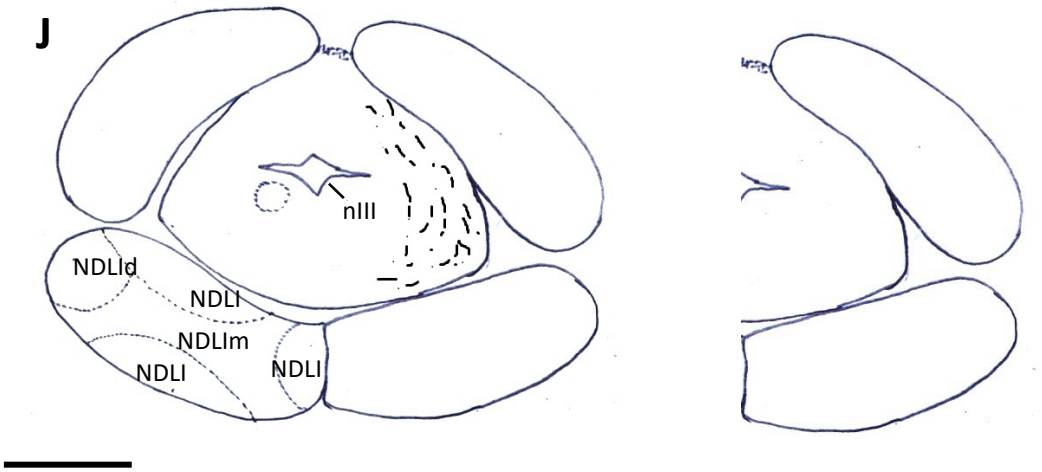

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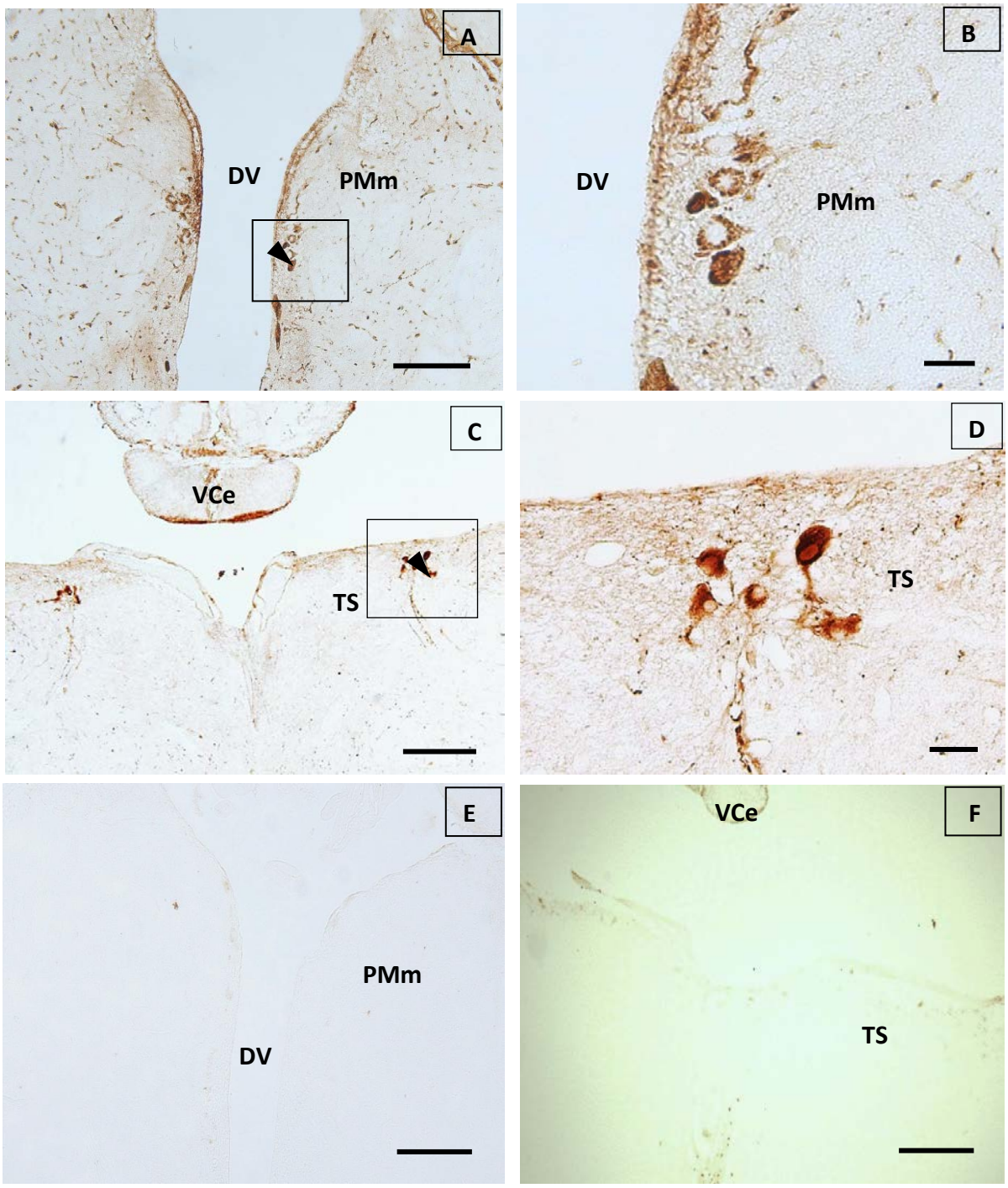

Fig. 3.2. Photomicrographs showing the localization of GnIH-ir cells in the brain of grass puffer. A-F: Photomicrographs of coronal sections with low (A and C) and high magnification (B and D) showing GnIH-ir cells in the magnocellular preoptic nucleus pars magnocelluaris

(PMm) and semicircular torus (TS). E and F: Pre-absorption with the antigen peptide (10 µg/µl) successfully eliminated GnIH immunoreactivity. DV, diencephalic ventricle; VCe, valvula of the cerebellum. Arrow heads indicate GnIH-ir cells and boxes direct higher magnification sites. Scale bars = 100 µm in A, C, E and F; 20 µm in B and D.

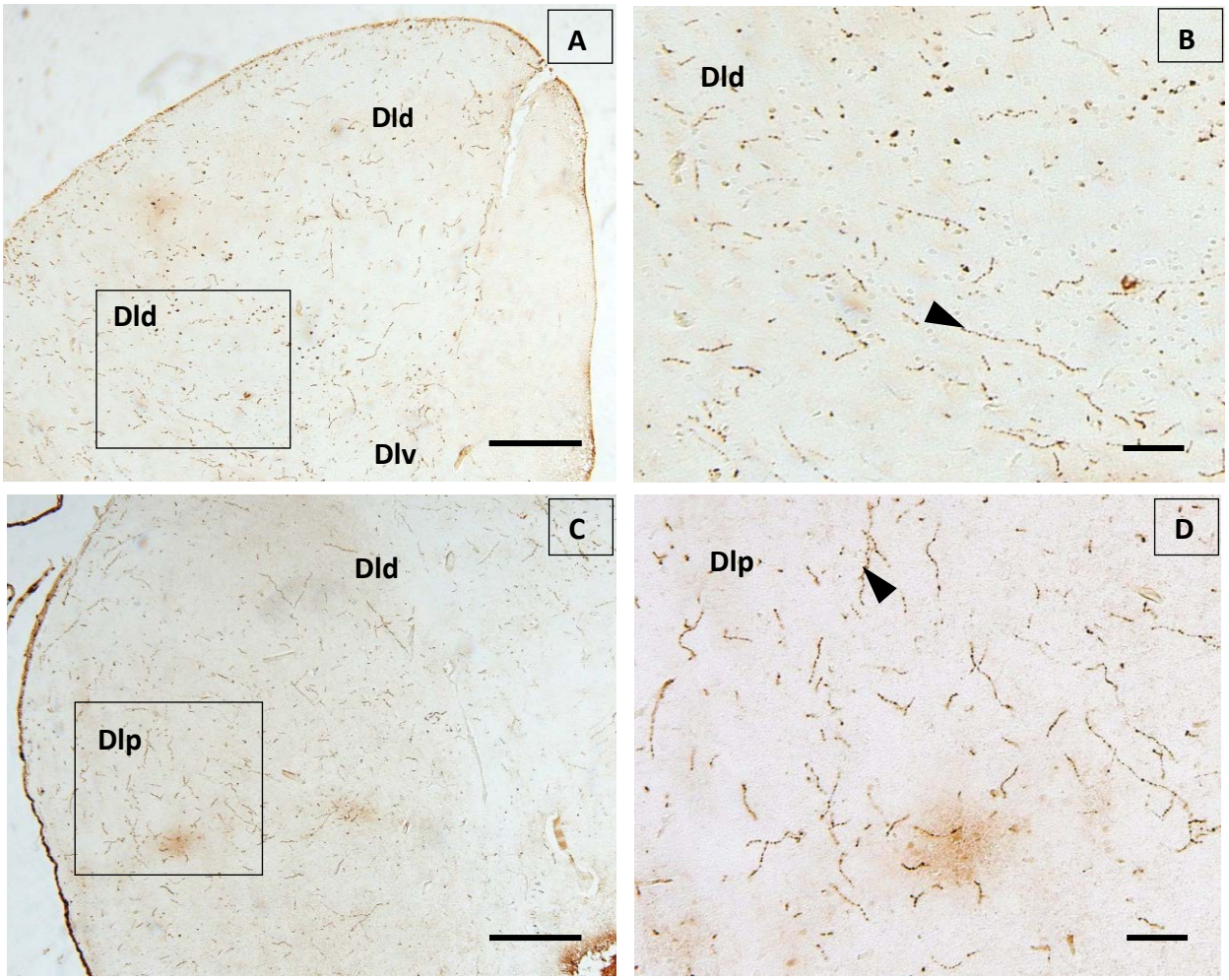

Fig. 3.3. Photomicrographs showing the distribution of GnIH-ir fibers in the brain of grass puffer. A-J: Photomicrographs of GnIH-ir fibers in the brain with low (A, C, E, G and I) and high (B, D, F, H and J) magnifications: the dorsal telencephalon (A-D), preoptic area (E and F) and optic tectum (G-J). Dld, Dorsal subdivision of the lateral part of the dorsal telencephalon; Dlp, posterior subdivision of the lateral part of the dorsal telencephalon; NAPv, Nucleus anterior periventricularis; CZ, central zone of the optic tectum; TP, Posterior tubercle. Arrow heads and box indicates GnIH-ir fibers and the area for higher magnifications, respectively. Scale bars = 100 µm in A, C, E, G and I; 10 µm in B, D, F, H and J.

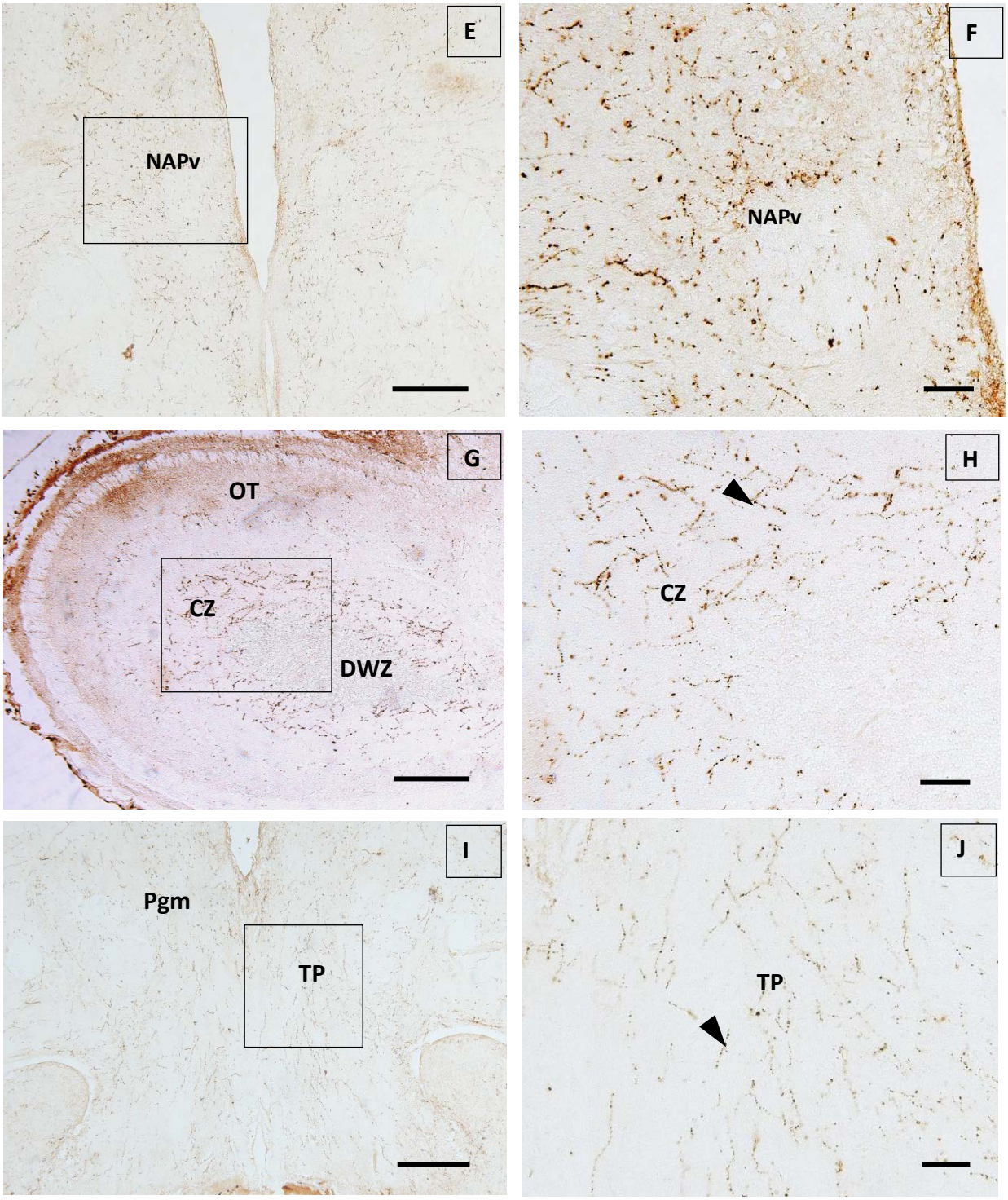

Fig. 3.3. (continued).

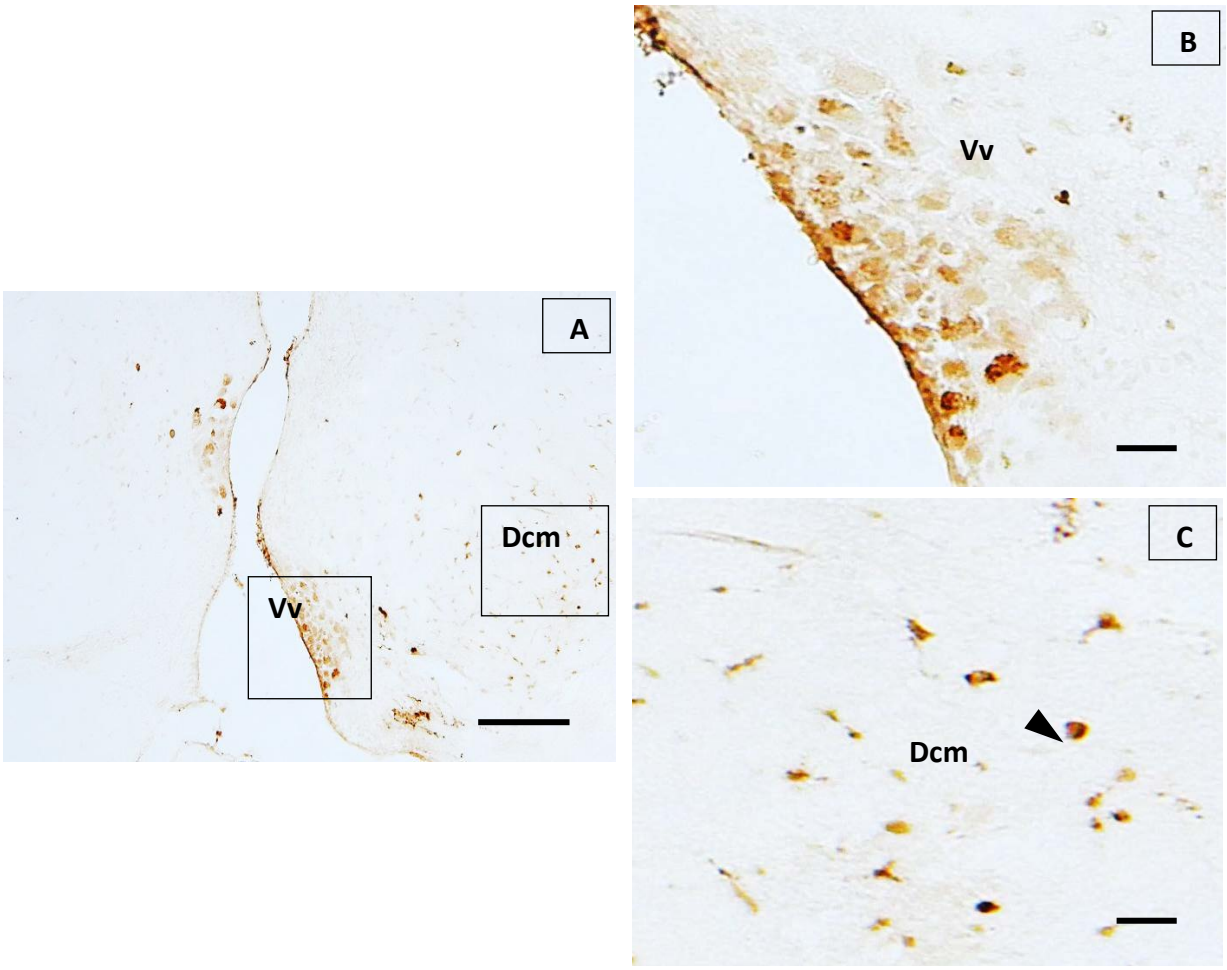

Fig. 3.4. Photomicrographs showing the localization of GnIH-R-ir cells in the brain of grass puffer. A-I: Photomicrographs of coronal sections showing GnIH-R-ir cells in the brain with low (A, D, F, H and I) and high (B, C, E and G) magnifications. Pre-absorption of anti-GnIH-R antiserum with the antigen peptide diminished the signals (H and I). A-G: GnIH-R-ir cells in the ventral part of the ventral telencephalon (Vv; A and B) and medial subdivision of central part of the dorsal telencephalon (Dcm; A and C), magnocellular preoptic nucleus pars magnocellularis (PMm; D and E) and medial preglomerular nucleus (Pgm; F and G). DV, Diencephalic ventricle; VCe, Valva of the cerebellum. Arrow heads indicate GnIH-R-ir cells and boxes direct higher magnification sites. Scale bars = 100 µm in A, D, F, H and I; 20 µm in B, C, E and G.

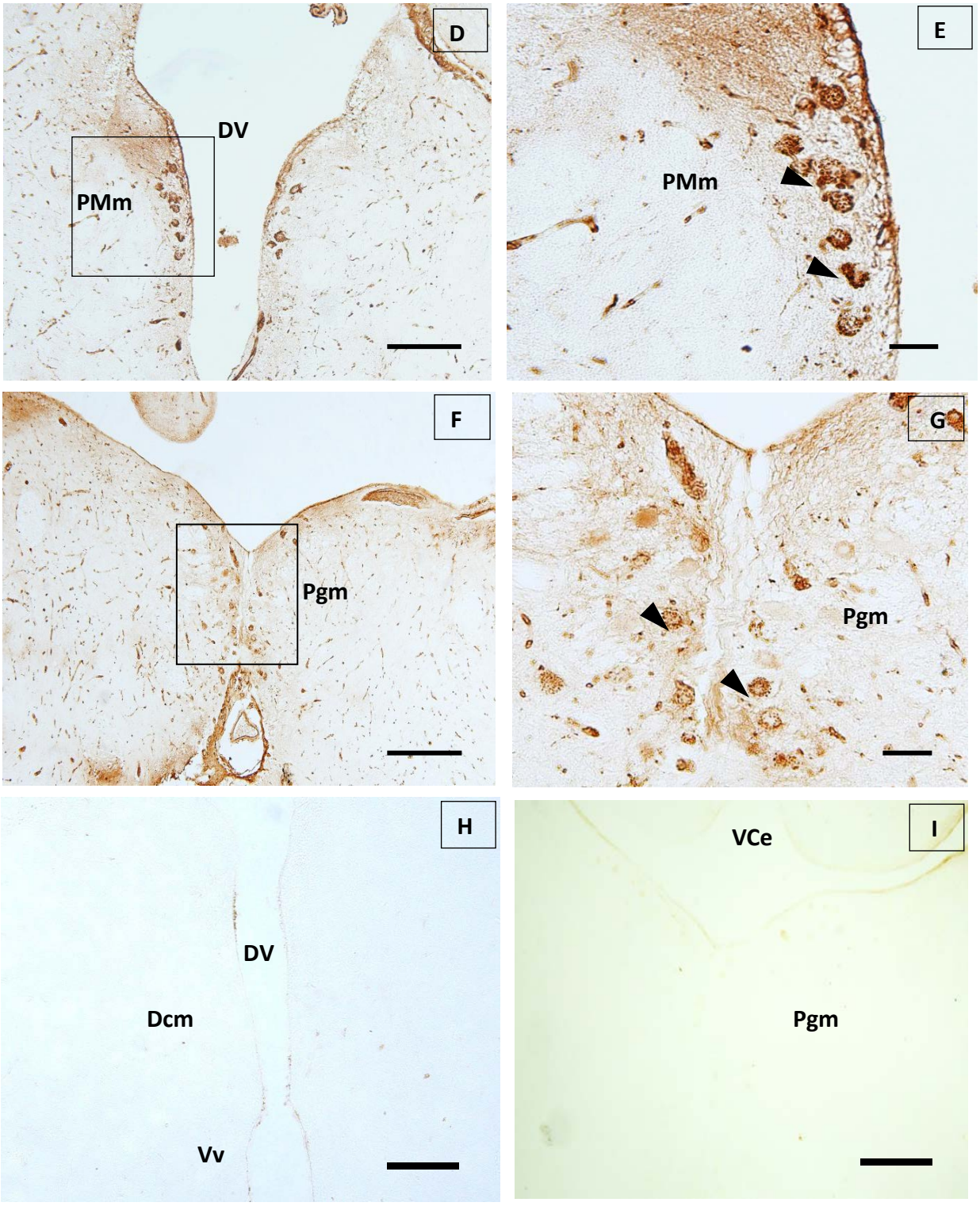

Fig. 3.4. (continued).

Chapter 4

Regulation of GnIH and GnIH receptor gene expression by water temperature

4.1. Introduction

The regulation of reproduction is governed by a complex interaction between environmental signals and various neurohormones including gonadotropin-releasing hormone (GnRH), kisspeptin and gonadotropin-inhibitory hormone (GnIH) secreted from particular neurons in the hypothalamus (Khan and Kauffman, 2012; Simonneaux et al., 2013). Water temperature has been considered as one of the most important environmental factors regulating the reproductive system in fish (Bromage et al., 2001; Wang et al., 2010; Pankhurst and Munday, 2011). Asymmetric fluctuation in water temperature can directly affects the different phases of fish reproductive cycle and both high and low water temperature has been shown to repress gonadal maturation in teleosts (Wang et al., 2010; Hermelink et al., 2011). However, the endocrine and neuroendocrine mechanisms underlying the impairment of reproductive function by water temperature remain unknown.

In some fish species, it has been shown that anomalous temperature affects the expression and function of hormones in the hypothalamic–pituitary–gonad (HPG) axis including GnRH, kisspeptin and GnIH in the hypothalamus, two gonadotropins (GTHs), namely, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the pituitary and gonadal steroid hormones. In Atlantic salmon, high water temperature disrupted the endocrine processes and consequently suppressed ovarian maturation, ovulation and successive fertility (King et al., 2007). GnRH-induced ovulation was inhibited with an increase in temperature in Chinese carps (Rottmann and Shireman, 1985). Furthermore, high temperature caused gonadal regression and associated decrease in plasma sex steroid concentration and reduced mRNA levels of the GTH subunit genes in the pejerrey (Soria et al., 2008) and red seabream (Okuzawa and Gen, 2013). On the other hand, a decrease in temperature also hampers

reproductive function in fish. Low water temperature showed limited yolk deposition, delayed ovulation and lowered fecundity in *Prochilodus argenteus* (Arantes et al., 2011). Therefore, it is acknowledged generally that increase and decrease in water temperature suppress fish reproduction through the impairment of various hormones in the HPG axis.

The grass puffer (*Takifugu alboplumbeus*) provides a unique and interesting animal model for studying the complex interaction between environmental signals and neuroendocrine systems regulating reproduction for its unique reproductive cycle, which is synchronized with seasonal, lunar and daily cycles (Ando et al., 2013). They breed seasonally from spring to early summer with the specialty of spawning that occurs only during spring tide every two weeks (Motohashi et al., 2010; Ando et al., 2013). On the day of spawning, spawning occurs 1.5–2 h before high tide and continues during the rising tidal phase (Motohashi et al., 2010; Ando et al., 2013). Therefore, spawning of the grass puffer is tightly connected with seasonal, lunar and tidal rhythms as well as daily rhythm where environmental factors such as light, tide, and water temperature play an important role in the control of reproduction (Ando et al., 2013). Recently, Shahjahan et al. (2017) showed that both low and high temperature conditions significantly decrease the expression levels of the genes encoding kisspeptin (*kiss2*), kisspeptin receptor (*kiss2r*) and *gnrh1* in the brain as well as *fshb* and *lhb* in the pituitary of the grass puffer. It was considered that the suppression of the *kiss2*/*kiss2r*/*gnrh1*/*fshb*/*lhb* expression pathway by the increase to 28 °C may lead to termination of reproduction at the end of breeding season in July in nature.

Besides the kisspeptin/GnRH/GTH systrm, GnIH, first identified in the Japanese quail (*Coturnix japonica*) as a suppressor of LH release (Tsutsui et al., 2000) has gained much attention for the neuroendocrine regulation of reproduction in fish. However, several evidences suggest controversial dual roles of GnIH. GnIH showed both stimulatory and inhibitory effects on GTH synthesis and release depending on species and gonadal stage (Amano et al., 2006; Zhang et al., 2010; Moussavi et al., 2012; Qi et al., 2013; Biran et al., 2014; Wang et al., 2015; Di Yorio et al., 2016; Paullada-Salmeron et al., 2016a). In the grass puffer, the expressions of *fshb* and *lhb* were stimulated by the goldfish LPXRFamide peptide-

1 (gfLPXRFa-1) administration (Shahjahan et al., 2011), suggesting that GnIH is a stimulatory hypothalamic neurohormone as well as GnRH in the grass puffer (Shahjahan et al., 2011; Ando et al., 2013).

Another important role of GnIH is to regulate the secretion of growth hormone (GH) and prolactin (PRL) from the pituitary, differentially depending on reproductive stage and fish species. In sockeye salmon, gfLPXRFa peptides increased the release of GH but PRL from the cultured pituitary cells (Amano et al., 2006). gfLPXRFa-3 showed inhibitory effects on basal and GnRH-induced GH release from the goldfish pituitary and these effects were seasonally dependent (Moussavi et al., 2014). In the grass puffer, gfLPXRFa-1 was found to have stimulatory effects on the expressions of *gh* and *prl* as well as *fshb* and *lhb* (Shahjahan et al., 2016). It is therefore conceivable that GnIH may play as a multifunctional hypophysiotropic neurohormone in the different fish species depending on their particular endocrine mechanism of reproduction.

For GH and PRL, both has been shown to be involved in the gonadal development and steroidogenesis in fish (Canosa et al., 2007; Reinecke, 2010; Whittington and Wilson, 2013). GH stimulated cell proliferation of spermatogonia in rainbow trout (Loir, 1999). GH stimulated steroid production in the testis and ovary of *Fundulus heteroclitis* (Singh et al., 1988). It also enhanced GTH-stimulated steroidogenesis in the goldfish ovary (Van der Kraak et al., 1990). On the other hand, PRL has been also shown to stimulate steroid production in guppy (Tan et al., 1988) and Nile tilapia (Rubin and Specker, 1992). The levels of PRL gene expression were higher in mature fish when compared to juvenile fish in blue gourami (Degani et al., 2010).

Accordingly, taking into account that the GnIH/GH/PRL system also participates in the control of reproduction, the effects of low and high temperature on the expression of genes for GnIH (*gnih*) and GnIH receptor (*gnihr*), along with *gh* and *prl*, were examined in this study to clarify the role of the GnIH/GH/PRL system in the temperature regulation of reproduction of the grass puffer. Here, the brain and pituitary samples of the previous study

(Shahjahan et al., 2017) were used in the present study to directly compare the effects on the the GnIH/GH/PRL system and the kisspeptin/GnRH/GTH system.

4.2. Materials and methods

4.2.1. Fish

Wild mature male grass puffer were caught at spawning grounds in Sado, Niigata, Japan in June for the 1st experiment, and in Kawana, Shizuoka, Japan in July for the 2nd experiment. The fish were transferred to the Marine Biological Station, Sado Island Center for Ecological Sustainability, Niigata University, Japan, and reared in indoor tanks (500 L) with flow of seawater under natural photoperiod (LD 14:10) for two weeks. The water temperature during the acclimatization period was similar to that of sampling sites, which was 18°C in the 1st experiment and 21°C in the 2nd experiment. They were fed commercial pellets equivalent to 1% of body weight (BW) daily. BW and gonadosomatic index (GSI = gonad weight/BW × 100) of the fish are shown in Table 4.1.

4.2.2. Experimental design and sample collection

Two experiments were conducted as described in detail by Shahjahan et al. (2017). In brief, in the 1st experiment (preliminary) in June, the fish were exposed to low temperature (11°C), normal temperature (18°C) and high temperature (25°C) for 3 and 7 days, which served as Day-3 and Day-7 groups, respectively (n = 5 for each group). To acclimatize fish to the temperature change, temperature was gradually decreased or increased (Δ1°C per 12 h) from normal to the target temperature conditions. In addition, fish sampled just before the temperature change served as initial control (IC) group. In the 2nd experiment in July, the fish were exposed to low temperature (14°C), normal temperature (21°C) and high temperature (28°C) for 7 days, and the fish of Day-7 and IC groups were sampled (n = 8).

The fish were anesthetized in 0.03% MS222 (Sigma–Aldrich, Tokyo, Japan), and BW and BW were measured to calculate GSI. The brains and pituitaries were removed and

soaked in RNAlater (Ambion, Austin, TX) at 4°C for 20 h. Then, the brains were trimmed to prepare diencephalon/midbrain samples, and they were stored at -80°C until extraction of total RNA. The experimental procedures followed the guidance approved by the Institutional Animal Care and Use Committee of Niigata University, Niigata, Japan.

4.2.3. Real-time PCR assay of *gnih, gnihr, gh* and *prl* mRNAs

Real-time PCR assay was carried out as described previously (Shahjahan et al., 2011, 2016). Briefly, total RNA was extracted from the diencephalon/midbrain and pituitary, and 200 ng of the total RNA was used for the synthesis of first strand cDNA by reverse transcription reaction using Multiscribe Reverse Transcriptase (Applied Biosystems, USA) according to the manufacturer's instruction. PCR reaction mixture (10 µl) contained 2 µl of sample cDNA, 0.2 µM of forward and reverse primers (Table 4.2) and 5 µl of SYBR Premix DimerEraser (Takara, Ohtsu, Japan). Amplification was carried out at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 60°C for 20 s. Specific amplification of each cDNA was verified by melting curve analysis and gel electrophoresis of the product. The slope and correlation coefficient (r) of the standard curves and intra- and inter-assay CVs in each assay are shown in Table 4.3.

3.2.4. Statistical analysis

The mRNA values are expressed as means ± standard error of the mean (SEM). Data were analyzed by ANOVA followed by Tukey's post hoc test to assess statistically significant differences among the different temperature conditions. Statistical significance was set at $P < 0.05$ or 0.01. Statistical analyses were performed using SPSS Version 14.0 for Windows (SPSS Inc., Chicago, IL).

4.3. Results

4.3.1. Changes in expression of *gnih* in the brain in the 1st experiment

In the 1st experiment, no distinct changes in GSI were observed in the experimental fish under different temperature conditions of Day-7 groups and they all showed similar GSI with IC group (Table 4.1). The amounts of *gnih* mRNA in the brain showed no noticeable changes in Day-3 and Day-7 groups, although there was a trend toward increased *gnih* mRNA in the high temperature (25°C) conditions compared to the normal temperature (18°C) conditions (Fig. 4.1).

4.3.2. Changes in expression of *gnih* and *gnihr* in the brain and pituitary in the 2nd experiment

In the 2nd experiment, GSI significantly decreased both in the low (14°C) and high temperature (28°C) when compared to the normal temperature (21°C) of Day-7 group and IC group (Table 4.1). The amounts of *gnih* and *gnihr* mRNAs in the brain were significantly decreased in the low (14°C) and high temperature (28°C) when compared to the normal temperature (21°C) of Day-7 group and IC group (Figs. 4.2A and 4.2B). Similarly, the amounts of *gnih* and *gnihr* mRNAs in the pituitary were decreased in the low and high temperature groups when compared to the group maintained at 21^{o}C and IC group, although the change in the high temperature group was not statistically significant (Figs. 4.3A and 4.3B).

4.2.3. Changes in expression of *gh* and *prl* in the pituitary in the 2nd experiment

Significantly lower amounts of *gh* mRNA were observed both in the low and high temperature groups compared to the normal temperature group and IC group (Fig. 4.4A). Unlike *gh*, the amounts of *prl* mRNA significantly lower in the low temperature group but not in the high temperature group when compared to the normal temperature group and IC group (Fig. 4.4B).

4.4. Discussion

Water temperature has been shown to have enormous effects on reproduction in fish. In the present study, the effects of low and high temperature on the expression of *gnih* and *gnihr* were investigated in the brain and pituitary and *gh* and *prl* in the pituitary of sexually mature male grass puffer. The *gnih*, *gnihr* and *gh* mRNA levels were significantly decreased by the exposure to low and high temperature conditions along with gonadal regression. On the other hand, while significant suppression of *prl* expression was observed in the low temperature conditions, *prl* mRNA levels did not change in the high temperature conditions. The present results suggest that GnIH, in cooperation with GnRH and kisspeptin, may play an important role in the control of reproduction according to the seasonal changes in water temperature particularly at the termination of breeding season and GH may also participate in this regulation as well as FSH and LH in the grass puffer.

In the 1st experiment in June, the level of *gnih* mRNA did not show any significant changes under low and high temperature conditions at Day-3 and Day-7 but tended to increase in the high temperature condition (25 °C) at Day-7 (Fig. 4.1). The GSI did not vary among different groups accordingly. In the 2nd experiment in July using the fish that were sexually more advanced (GSI of the IC group: 10.0% in the 1st experiment and 11.9% in the 2nd experiment), the *gnih* mRNA levels significantly decreased in both low (14 °C) and high (28 °C) temperature conditions at Day-7 concurrently with the decrease in the GSI (Fig. 4.2A). Since the breeding season of grass puffer starts in May at about 16 °C of water temperature and terminates at the end of July at about 28 °C in wild conditions, the low temperature condition both in 1st (11 °C) and 2nd (14 °C) experiment could keep fish under stress before starting breeding while the high temperature conditions at 25 °C in the 1st experiment may be acceptable for fish to progress sexual maturation and the *gnih* expression may remain augmented. Conversely, high temperature at 28 °C in the 2nd experiment may be transmitted to the hypothalamus as an environmental cue that terminates reproduction and inhibit the expressions of *gnih* and associated genes in the GnIH/GH system.

GnIH has been shown to have stimulatory effects on the regulation of reproduction in the grass puffer: the administration of sexually matured fish with gfLPXRFa-1 stimulated the

expressions of *fshβ*, *lhβ* and *gh* in vivo (Ando et al., 2018) and in vitro (Shahjahan et al., 2011, 2016). The previous study on the effects of low and high temperature on the genes for the kisspeptin/GnRH/FSH/LH system clearly showed that the expressions of *kiss2*, *kiss2r*, *gnrh1*, *fshβ* and *lhβ*, but not *gnrh2* and *gnrh3*, decreased under the low (14°C) and high (28°C) temperature conditions (the 2nd experiment, Shahjahan et al., 2017). Therefore, these results suggest that GnIH may interact with GnRH1 and/or kisspeptin to regulate FSH and LH secretion. In addition, previous studies showing coordinated expressions of *gnih*, *gnihr*, *kiss2*, *kiss2r* and *gnrh1* in regard to seasonal, daily and circadian variations in grass puffer further support this notion (Ando et al., 2013, 2014, 2018; Shahjahan et al., 2010a, b, 2011). The present study is the first report demonstrating the effects of temperature changes on *gnih* and *gnihr* expressions in mature fish, particularly in the fish where GnIH has a stimulatory role in the control of reproduction. It is of considerable interest and importance to determine whether *gnih* and *gnihr* expression is suppressed or stimulated by temperature changes in the fish where GnIH negatively regulates reproduction.

Neuroanatomical relationship of GnIH neurons and GnRH neurons has been examined in several fish species. In tilapia, there was no close association of GnIH-immunoreactive (ir) fibers and GnRH1 and GnRH3 neurons (Ogawa et al., 2016). In the South American cichlid fish, although no apparent contacts between GnIH and GnRH1 neurons were observed, colocalization of GnIH and GnRH3 was found in the nucleus olfacto-retinalis (Di Yorio et al., 2019). Moreover, in zebrafish, GnIH-ir fibers project to and contact with the hypophysiotropic GnRH3-ir neurons in the forebrain region (Spicer et al., 2017). Thus, it is possible that GnIH regulates the expression of GTH genes in the pituitary directly and indirectly through interaction with GnRH1 neurons. In the grass puffer, GnIH-ir cells were found to be localized in two regions, the magnocellular preoptic nucleus and semicircular torus and the GnIH-ir fibers were widely distributed in the forebrain, midbrain and hindbrain (Chapter 3, Table 3.2, Figs. 3.1, 3.2 and 3.3). However, the neuroanatomical interaction of GnIH and GnRH soma and fibers remain to be determined.

The expression of *gh* was significantly suppressed under the low and high temperature conditions, while the *prl* mRNA levels was considerably decreased by the low temperature but not by high temperature conditions (Figs. 4.4A and 4.4B). The previous study showed significant decreases in *fshβ* and *lhβ* expressions under the low and high temperature conditions (the 2nd experiment, Shahjahan et al., 2017). Similar concomitant decreases in the *gh* mRNA levels with *fshβ* and *lhβ* mRNAs were observed in the female blue gourami exposed to low and high temperature (Levy et al., 2011). In addition, negative effects of high temperature on the *fshβ* and *lhβ* mRNA levels were reported in red seabream (Okuzawa and Gen, 2013). These results clearly indicate negative effect of anomalous temperature on the reproductive activity of teleost fishes through suppression of *gh* as well as *fshβ* and *lhβ* expressions. Taking into account that the *gh* expression is extensively activated during the breeding season possibly through the stimulation by GnIH (Shahjahan et al., 2016) and direct evidences showing stimulatory roles of GH in gonadal maturation and steroidogenesis (Hu et al., 2019; Singh et al., 1988; Van der Kraak et al., 1990), GH may have an important role in gonadal maturation during the breeding season in the grass puffer.

Although PRL has been shown to be involved in gonadal development and steroidogenesis and its levels are affected by GnRH and gonadal steroid (Whittington and Wilson, 2013), *prl* showed different expression patterns from *gh* and GTH subunit genes in the present study. The present results lead us to presume that *prl* expression may be related to stress under the low temperature conditions for the plasma cortisol levels were significantly increased under the low temperature conditions but not under the high temperature conditions (Shahjahan et al., 2017). In fish, it is acknowledged generally that PRL and cortisol are important in osmoregulation, in adaptation to freshwater (FW) and seawater (SW) environments, respectively (McCormick, 2001; Seale et al., 2013). The relative importance of PRL in FW and cortisol in SW adaptation, respectively, agrees with the inhibition of PRL secretion by cortisol. Hypoosmotically-induced PRL release was inhibited following exposure to cortisol in tilapia (Borski et al., 1991). Cortisol also directly suppressed the expressions of two PRL genes in the tilapia pituitary (Uchida et al., 2004).

These facts suggest that the suppression of *prl* expression seems to be due to high levels of plasma cortisol and the low temperature conditions may cause stress in this species.

Table 4.1. Body weight and gonado-somatic index of the fish used in the present study.

		Initial control	Day-7		
1st exp.		18°C	11°C	18°C	25°C
	BW (g)	37.7 ± 5.8[a]	37.3 ± 4.3[a]	42.5 ± 6.8[a]	37.3 ± 6.7[a]
	GSI (%)	10.0 ± 1.4[a]	10.0 ± 1.0[a]	10.5 ± 0.5[a]	9.6 ± 1.2[a]
	n	5	5	5	5
2nd exp.		21°C	14°C	21°C	28°C
	BW (g)	66.9 ± 3.6[a]	59.9 ± 3.1[a]	68.0 ± 5.2[a]	59.2 ± 4.8[a]
	GSI (%)	11.9 ± 1.1[a]	7.1 ± 0.4[b]	12.4 ± 1.1[a]	5.8 ± 0.8[b]
	n	8	8	8	8

[a,b] Values with different characters are significantly different among groups. Data are shown as the means ± SEM.

Table 4.2. Primers used in real-time PCR.

Primers	Nucleotide sequences
tnGnIH-qPCR-F1	5′-TGATTCGTCTGTGCGAGGAC-3′
tnGnIH-qPCR-R1	5′-TCAGCAGCTGTGCATTGACC-3′
tnGnIH-R-qPCR-F1	5′-AAGATGCTCATCCTGGTGGC-3′
tnGnIH-R-qPCR-R1	5′-AGATCCACCTGGTCACTGTCC-3′
tnGH-qPCR-F1	5′-TTCGGTCCTCAAGCTATTGTCC-3′
tnGH-qPCR-R1	5′-GCTCCTCTCCTCCCAGATTTTG-3′
tnPRL-qPCR-F1	5′-ACAGTGGAGGCAATGACTTGG-3′
tnPRL-qPCR-R1	5′-CTCTGGTACCATGTTTGCCATC-3′

Table 4.3. Slope and correlation coefficient (*r*) of standard curves and intra- and inter-assay coefficients of variation (CVs) in real-time PCR assays.

Genes	Slope	r	Intra-assay CV (%)	Inter-assay CV (%)
GnIH	-3.45 ± 0.0155	0.984 ± 0.001	7.11 ± 2.07	3.60
GnIH-R	-3.36 ± 0.0525	0.986 ± 0.002	6.46 ± 1.38	7.68
GH	-3.38 ± 0.1331	0.993 ± 0.001	6.04 ± 0.42	3.23
PRL	-3.39 ± 0.0105	0.995 ± 0.001	4.97 ± 1.18	4.08

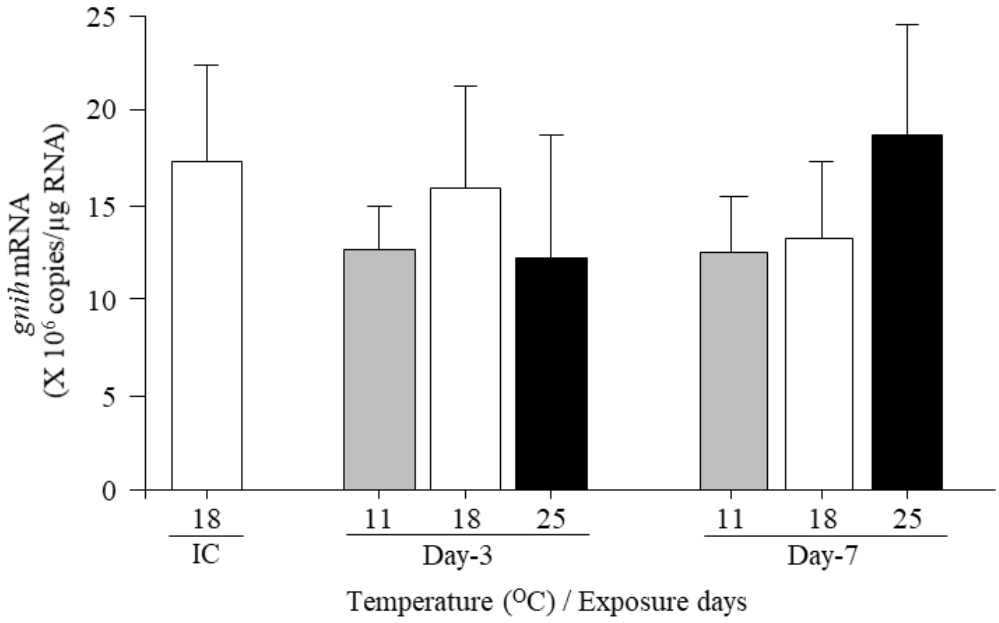

Fig. 4.1. Changes in the amounts of *gnih* mRNA in the brain of male grass puffer exposed to a low temperature (11°C), normal temperature (18°C), and high temperature (25°C) for 3 and 7 days. Before starting to exposure to the target temperature conditions, fish were sampled to obtain baseline values which served as initial control (IC). Values represent the mean ± SEM (n = 5).

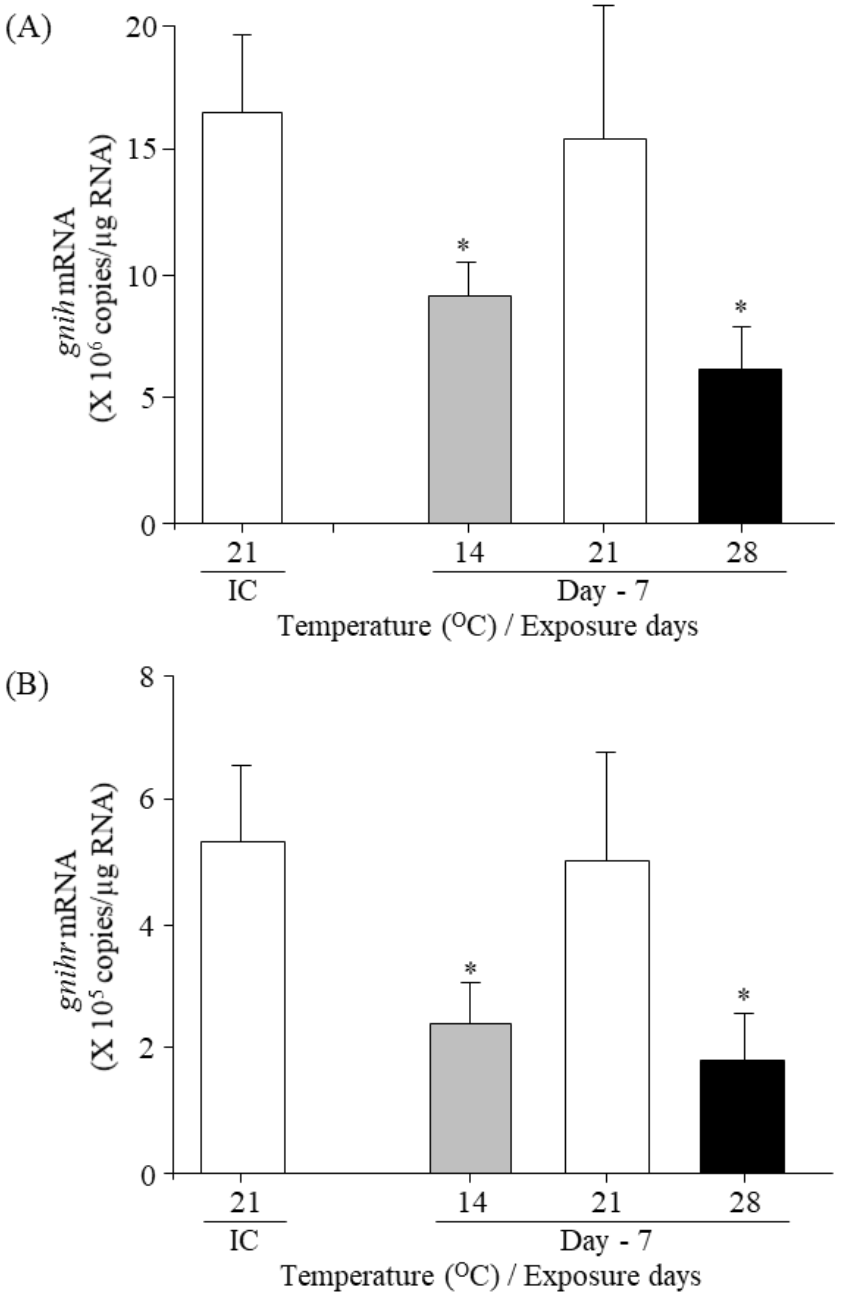

Fig. 4.2. Changes in the amounts of *gnih* (A) and *gnih-r* (B) mRNAs in the brain of male grass puffer exposed to a low temperature (14°C), normal temperature (21°C) and high temperature (28°C) for 7 days. Before starting exposure to the target temperature conditions, fish were sampled to obtain baseline values which served as initial control (IC). Values represent the mean ± SEM (n = 8). Asterisk indicates a significant difference in the low and high temperature groups compared to the normal temperature group (*, $P < 0.05$).

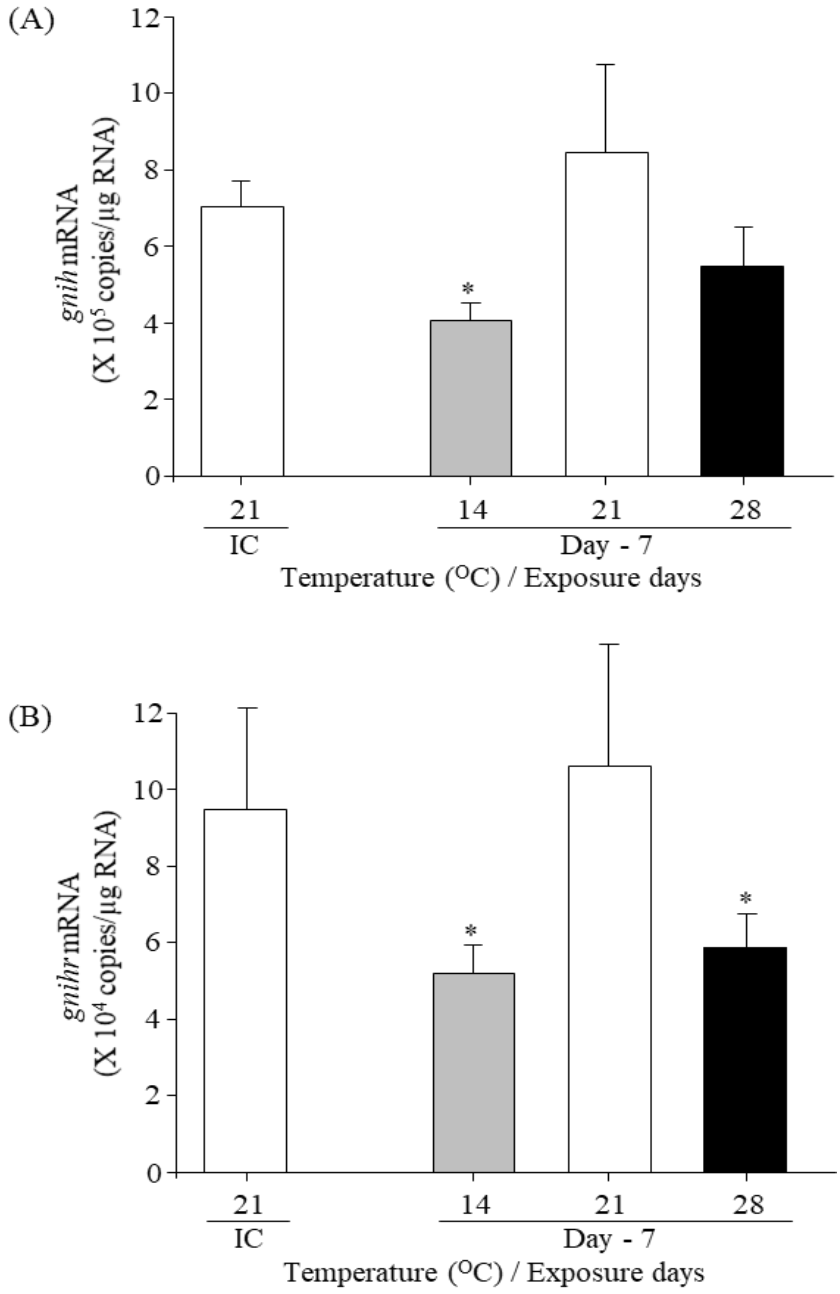

Fig. 4.3. Changes in the amounts of *gnih* (A) and *gnih-r* (B) mRNAs in the pituitary of male grass puffer exposed to a low temperature (14°C), normal temperature (21°C) and high temperature (28°C) for 7 days. Before starting to exposure to the target temperature conditions, fish were sampled to obtain baseline values which served as initial control (IC). Values represent the mean ± SEM (n = 8). Asterisk indicates a significant difference in the low and high temperature groups compared to the normal temperature group (*, $P < 0.05$).

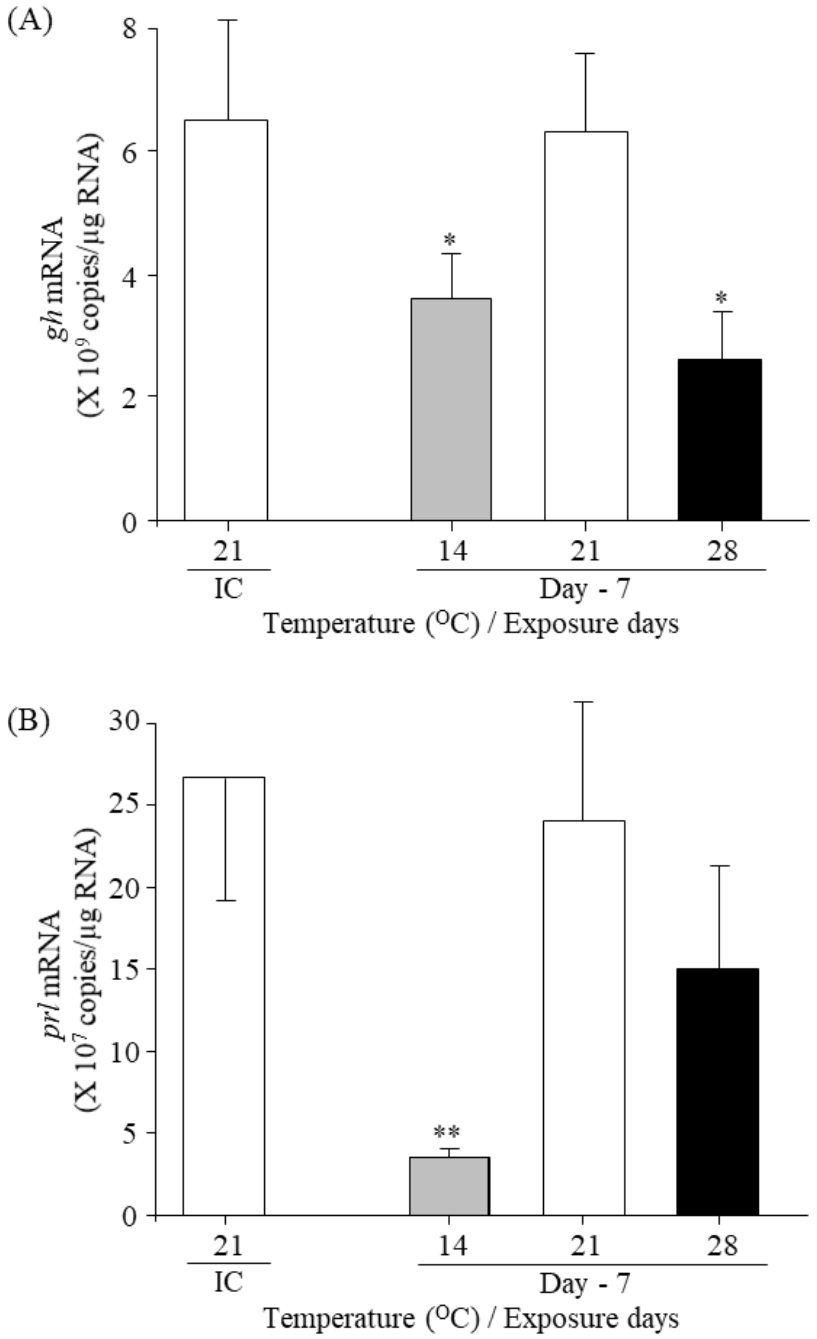

Fig. 4.4. Changes in the amounts of *gh* (A) and *prl* (B) mRNAs in the pituitary of male grass puffer exposed to a low temperature (14°C), normal temperature (21°C) and high temperature (28°C) for 7 days. Before starting to exposure to the target temperature conditions, fish was sampled to obtain baseline values which served as initial control (IC). Values represent the mean ± SEM (n = 8). Asterisk indicates a significant difference in the low and high temperature groups compared to the normal temperature group (*, $P < 0.05$; **, $P < 0.01$).

Chapter 5

Lunar-age dependent oscillations in expression of genes for kisspeptin, GnIH and their receptors

5.1. Introduction

Most vertebrate reproductive success is dependent on the synchronization of recruitment, development and release of their gametes, and synchronous reproductive behavior is an important adaptive strategy that enables higher reproductive efficiency, less predation risk and the prevention of interspecies mating (Domeier and Colin, 1997). Environmental cues such as temperature, moonlight and tidal changes have been proved to trigger initiation and synchronization of various reproductive functions of many organisms. Variations in the environmental signals are often used by fish as a forcing oscillation which entrains a biological rhythm (often called a Zeitgeber) for synchronization of reproductive activity. These signals from environmental parameters are integrated to regulate the activity of the hypothalamic-pituitary-gonadal (HPG) axis. In the hypothalamus, multiple neuropeptides such as gonadotropin-releasing hormone (GnRH), kisspeptin and gonadotropin-inhibitory hormone (GnIH) participate in the control of reproduction. Neurons producing these neuropeptides are periodically controlled by complex networks integrated by environmental and endocrine signal mechanisms, which are still urging a clear understanding.

Kisspeptin plays an important role in the control of reproduction in fish for it regulates the secretion of two gonadotropins (GTHs), namely, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary (Parhar et al., 2004; Chang et al., 2012; Espigares et al., 2015). Multiple forms of kisspeptin genes (*kiss1* and *kiss2*) and kisspeptin receptor genes (*kissr1* and *kissr2*) have been identified in several fish species (Felip et al., 2009; Kitahashi et al., 2009; Lee et al., 2009; Li et al., 2009; Mechaly et al., 2010), while some fish species including grass puffer have only single kisspeptin (*kiss2*) and its receptor

(*kissr2*) genes (Shahjahan et al., 2010b; Shi et al., 2010). A number of studies suggested that among the two forms of kisspeptin, Kiss2 is more potential to control reproduction in fish (Felip et al., 2009; Kitahashi et al., 2009; Shahjahan et al., 2010b; Zmora et al., 2012; Shahjahan et al., 2013; Espigares et al., 2015). It has been proposed that kisspeptin plays an important role in transmitting multiple environmental and endocrine signals such as time, photoperiod, energy status, stress and sex steroid hormones (Parhar et al., 2012; Simonneaux and Bahougne, 2015). However, the molecular mechanisms of the periodic regulation of kisspeptin neurons are mostly unknown.

GnIH is a multifunctional neuropeptide that also plays an important role in reproduction regulating the secretion of GTHs (Tsutsui, 2010; Ogawa and Parhar, 2014). It is now established that reproductive development and maintenance are be regulated by GnIH pathway in many vertebrate species (Ubuka et al., 2003; Quennell et al., 2010; Poling et al., 2012; Iwasa et al., 2012; Zhao et al., 2014). In fish, GnIH showed both stimulatory and inhibitory effects on GTH synthesis and release depending on species and gonadal stage (Amano et al., 2006; Zhang et al., 2010; Shahjahan et al., 2011; Moussavi et al., 2012; Qi et al., 2013; Biran et al., 2014; Wang et al., 2015; Di Yorio et al., 2016; Paullada-Salmerón et al., 2016a). In the grass puffer, the expressions of *fshb* and *lhb* were stimulated by the goldfish LPXRFamide peptide-1 (gfLPXRFa-1) administration (Shahjahan et al., 2011), suggesting that GnIH has a stimulatory role in the control of reproduction in the grass puffer.

The grass puffer (*Takifugu alboplumbeus*) shows unique reproductive physiology that is synchronized with seasonal, lunar and daily cycles. In early summer from May to July, they aggregate at certain seashore place for spawning 2–3 h before high tide at dusk only during spring tide every two weeks, and then spawning occurs 1–2 h before high tide (Motohashi et al., 2010; Ando et al., 2013). Therefore, the timing of spawning is tightly connected with lunar and tidal rhythms as well as daily rhythm. Previous studies revealed that *kiss2*, *gnih* and their receptor genes in the brain show seasonal variations during reproductive cycle with massive expression during the breeding season (Shahjahan et al., 2010b, 2011; Ando et al., 2013). Moreover, during the breeding season, *kiss2*, *gnih* and their receptor genes also show

daily and circadian oscillations in expression in association with the expression of melatonin receptor genes (Ikegami et al., 2009; Shahjahan et al., 2011; Ando et al., 2014). These results suggest that the expression of these genes may be under common regulatory mechanisms with respect to daily and circadian oscillations, possibly through the circadian clock and melatonin. For the periodic regulation of kisspeptin and GnIH, the circadian clock and melatonin have been shown to transmit the daily, circadian and seasonal information to kisspeptin and GnRH neurons in the hypothalamus in birds and mammals (Khan and Kauffman, 2012; Williams and Kriegsfeld, 2012; Simonneaux et al., 2013).

Among various biological rhythms, circalunar rhythm is approximately one month of cycle which plays an important role in the synchronization of reproduction in many organisms. Lunar- and tidal cycle-synchronized reproduction has been reported in a wide variety of marine organisms, particularly in teleost fish that subsist in coral and shallow area (Hsiao and Meier, 1989; Sadovy et al., 1994; Takemura et al., 2004a; Wang et al., 2008; Motohashi et al., 2010). In these organisms, changes in moonlight and tide are considered to act as environmental cues that entrain biological clocks which have been proposed as circalunar and circatidal clocks, respectively. However, in contrast to the molecular mechanisms of daily and circadian rhythms that have been the focus of considerable attention, those of lunar and tidal cycle-synchronized rhythms are little understood. Taking into account that the genes for kisspeptin, GnIH and their receptors are under control of the circadian clock and melatonin in the grass puffer, the kisspeptin/GnIH system may play an important role in the regulation of the lunar-synchronized spawning, although the molecular mechanisms of the circalunar and circatidal clocks and their molecular and functional interaction with the kisspeptin/GnIH system remain to be determined.

In the present study, as a first step to elucidating the mechanism of lunar-synchronized spawning rhythm of the grass puffer, the changes in expression of the genes for the kisspeptin/GnIH system were examined if these genes are regulated by lunar cycle during the breeding season. Since their expression levels show daily oscillations (Shahjahan et al.,

2011; Ando et al., 2014), the lunar-age dependent changes in expression were examined at daytime or nighttime during the breeding season in different years.

5.2. Materials and methods

5.2.1. Fish

Wild sexually mature male grass puffer were caught at Shikanoshima Island, Fukuoka, Japan, in May 2009 and 2010. They were transferred to the Fishery Research Laboratory Station, Kyushu University, Fukutsu, Japan. The fish were transferred into indoor tanks (120 L) and acclimatized with flow of seawater (19-23°C) under natural photoperiod (LD 14:10) for one week. They were fed commercial pellets equivalent to 1% of BW daily until 3 days before sampling. Body weight (BW) and gonadosomatic index (GSI = gonad weight/BW × 100) of the fishes were 64.4 ± 1.9 g and 20.5 ± 0.2% in 2009, and 64.4 ± 0.2 g and 19.2 ± 0.5% in 2010, respectively.

5.2.2. Experimental design and sample collection

The fish were anesthetized in 0.03% MS222 and killed by decapitation at Zeitgeber time (ZT) 9 (daytime) at four- or five-day interval on lunar age 25.0, 0.6, 5.6, 10.6, 14.6, 19.6 and 24.6 from May to June 2009 (n = 8 for each lunar age) and at ZT18 (nighttime) on lunar age 10.1, 15.1, 20.1, 25.1, 0.7, 5.7 and 10.7 from May to June 2010 (n = 8 for each lunar age). After decapitation, brains and pituitaries were removed and soaked in RNAlater (Ambion). The brains were trimmed to prepare the diencephalon sample, immediately frozen by liquid nitrogen and stored at –80°C until extraction of total RNA. The experimental procedures followed the guidance approved by the Institutional Animal Care and Use Committee of Kyushu University, Fukuoka, Japan.

5.2.3. Real-time PCR assay of *kiss2, kiss2r, gnih* and *gnihr* mRNAs in the brain and pituitary

Total RNA was extracted from the diencephalon and pituitary by using guanidinium thiocyanate–phenol–chloroform method and stocked at –80°C for further procedure after transfer to Sado Marine Biological Station, Niigata University, Japan. For real-time PCR assay, 200 ng of total RNA was used for the synthesis of first strand cDNA by reverse transcription reaction using Multiscribe Reverse Transcriptase (Applied Biosystems, USA) according to the manufacturer's instruction. The real-time PCR assay for *kiss2*, *kiss2r*, *gnih* and *gnihr* was carried out as described previously (Shahjahan et al., 2010a, b). PCR reaction mixture (10µl) contained 2µl of sample cDNA, 0.2µM of forward and reverse primers (Table 5.1) and 5µl of SYBR Premix Dimer Eraser (Takara, Ohtsu, Japan). Amplification was carried out at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 55°C for 30 s, and 72°C for 30 s. Specific amplification of each subtype cDNA was verified by melting curve analysis and gel electrophoresis of the product.

5.2.4. Statistical analysis

The mRNA values are expressed as means ± standard error of the mean (SEM). Data were analyzed by ANOVA followed by Tukey's post hoc test to assess statistically significant differences among the different day points in the monthly variation. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using SPSS Version 14.0 for Windows (SPSS Inc., Chicago, IL).

5.3. Results

5.3.1. Lunar-age dependent oscillations in expression of *kiss2* and *kiss2r* in the brain and pituitary

Lunar-age dependent expressions of *kiss2* and *kiss2r* were examined in the diencephalon by real-time PCR. The brain samples were taken at ZT9 during the daytime and ZT18 during the nighttime every four or five days for a lunar month during the breeding season. The expression levels of *kiss2* were different between ZT9 and ZT18, being about 8-fold higher

the peak levels at ZT18 compared to ZT9 (Fig. 5.1A). However, at both time of sampling, the expression levels showed significant lunar-age dependent variations with one peak at lunar age 10 at ZT9 and lunar age 15 at ZT18. The expression patterns of *kiss2r* were similar to those of *kiss2* during the lunar cycle (Fig. 5.1B).

In the pituitary, the expression levels of *kiss2* at ZT18 were observed to be about two-fold greater compared to ZT9. The expression levels showed lunar-age dependent variations during the lunar month with one peak at lunar age 10 at ZT9 and lunar age 15 at ZT18, though the change at ZT9 was not statistically significant (Fig. 5.2A). The expression patterns of *kiss2r* were similar to *kiss2* with a significant increase at lunar age 15 at ZT18 (Fig. 5.2B).

5.3.2. Lunar-age dependent oscillations in expression of *gnih* and *gnihr* in the brain and pituitary

In the brain, the expression levels of *gnih* showed significant lunar-age dependent oscillations at both ZT9 and ZT18, though the expression patterns were different between ZT9 and ZT18: there were two peaks at lunar age 0 and 20 at ZY9, while one peak at lunar age 0 at ZT18 (Fig. 5.3A). The peal levels of expression at ZT18 were about 2-fold higher than ZT9. The expression patterns of *gnihr* were basically similar to *gnih* during the lunar cycle (Fig. 5.3B).

In the pituitary, the expression patterns of *gnih* and *gnihr* were basically similar to those in the brain: two peaks at lunar age 0 and 20 at ZT9 and one peak at lunar age 0 at ZT18, though the change of *gnih* at ZT18 was not statistically significant (Figs. 5.4A and 5.4B).

5.4. Discussion

In the present study, to explore the role of kisspeptin/GnIH system in the regulation of lunar-synchronized spawning of the grass puffer, the lunar-age dependent variations in expression of *kiss2*, *kiss2r*, *gnih* and *gnihr* were examined in the diencephalon and pituitary

for a lunar month during the breeding season. The diencephalon and pituitary samples were taken at two different time points, ZT9 during the daytime and ZT18 during the nighttime, because all four genes showed daily oscillations in expression in the grass puffer (Shahjahan et al., 2011; Ando et al., 2014). In all the situations, in the diencephalon or pituitary and during the daytime or nighttime, all four genes showed lunar-age dependent oscillations in expression, though their expression patterns were different depending on the situations and some of the changes were not so clear as to be statistically significant. Nevertheless, the present results indicate that the expression of kisspeptin, GnIH and their receptors are highly dependent on the lunar age during the breeding season, suggesting that the action of kisspeptin/GnIH system on the HPG axis may change during the lunar month. It is therefore conceivable that the kisspeptin/GnIH system may play a pivotal role in the regulation of lunar-synchronized spawning of the grass puffer. Moreover, this is the first report of lunar-synchronized expression of genes for the kisspeptin/GnIH system.

The expression levels of *kiss2* and *kiss2r* in the brain substantially increased at ZT18 on the day of lunar age 15, i.e. full moon night, when spawning of the grass puffer occurs in the wild (Figs. 5.1A and 5.1B). These temporal increases were also observed in the pituitary (Figs. 5.2A and 5.2B). The massive expression of *kiss2* and *kiss2r* at full moon night may result in stimulating spawning behavior by directly activating the neural activity in the brain and induction of ovulation and spermiation by directly or indirectly (through GnRH1) stimulating the secretion of LH and FSH from the pituitary. During the daytime at ZT9, their expression levels were temporally increased at lunar age 10 but the maximum levels of expression were similar to the basal levels at ZT18, suggesting that the expression of *kiss2* and *kiss2r* is under the control of a lunar-age dependent mechanism both during daytime and nighttime and this control may be time- and/or light-sensitive and reinforced during the nighttime.

The expression patterns of *gnih* and *gnihr* in the brain were also lunar-age dependent at ZT9 and ZT18 but were different between the two time points and distinct from those of *kiss2* and *kiss2r*. During nighttime at ZT18, they showed a temporal increase at lunar age 0, i.e.

new moon night, when spawning of the grass puffer occurs in the wild (Figs. 5.3A and 5.3B). These temporal increases were also observed in the pituitary (Figs. 5.4A and 5.4B). Taking into account that GnIH has a stimulatory role in the control of reproduction in the grass puffer (Shahjahan et al., 2011), the massive expression of *gnih* and *gnihr* at new moon night may result in directly stimulating spawning behavior in the brain and induction of spawning by stimulating the GTH secretion like *kiss2* and *kiss2r*. Accordingly, the lunar-age dependent oscillations of these genes strongly support the notion that the kisspeptin/GnIH system plays a central role in the regulation of lunar-synchronized spawning of the grass puffer.

During the daytime at ZT9, the expression levels of *gnih* and *gnihr* showed two peaks during the lunar month at lunar age 0 and 20 in both the brain and pituitary (Figs. 5.3A, 5.3B, 5.4A and 5.4B). Based on these results and the different peaks of lunar age for *kiss2*/*kiss2r* (full moon) and *gnih*/*gnihr* (new moon) at ZT18, it is tempting to speculate that the lunar-age dependent mechanisms may be different between daytime and nighttime, possibly time- and/or light-sensitive, and between *gnih*/*gnihr* and *kiss2*/*kiss2r*. On the contrary, it should be also noted that the expression patterns were almost similar between the genes for ligand and receptor, that is, *kiss2* and *kiss2r*, *gnih* and *gnihr*. respectively. Moreover, the expression patterns were almost similar between the brain and pituitary. Taken together, the present results indicate that *kiss2*/*kiss2r* and *gnih*/*gnihr* may be differentially regulated by lunar-age dependent mechanisms that are time- and/or light-sensitive in both the brain and pituitary.

Lunar- and tidal cycle-synchronized reproduction has been reported in a wide variety of marine organisms (Hsiao and Meier, 1989; Sadovy et al., 1994; Takemura et al., 2004a; Wang et al., 2008; Motohashi et al., 2010). However, the molecular and neuroendocrine mechanisms through which the lunar/tidal cycles change the reproductive function remain unknown. One possible mechanism is the role of melatonin in the control of kisspeptin/GnIH system. Melatonin secretion from the pineal gland is dependent on time and light, only secreted during dark phase in many animals (Reiter, 1993). Thus, melatonin, “the nocturnal hormone” can transmit the information of day/night cycle and seasonal changes in photoperiod to the central and peripheral organs (Falcón et al., 2007). Melatonin regulation

of kisspeptin and GnIH expressions has been reported in seasonally breeding birds and mammals (Ubuka et al., 2005; Revel et al., 2008; Simonneaux et al., 2009). In the grass puffer, *kiss2*, *kiss2r*, *gnih* and *gnihr* show daily and circadian oscillations in expression, and four subtypes of melatonin receptor genes show daily and circadian expressions with similar peak time to the four genes (Ikegami et al., 2009; Ando et al., 2013), suggesting the regulation of the four genes by melatonin. Indeed, the involvement of melatonin and melatonin receptor in the control of lunar-synchronized reproduction has been reported in several fish species. In the golden rabbitfish, lunar-age dependent variations in plasma melatonin concentration was found to be critical for spawning at first quarter moon (Takemura et al., 2004b). The lunar-age dependent expression of melatonin receptor genes has been reported in the golden rabbitfish (Park et al., 2014) and mudskipper (Hong et al., 2014). It is therefore conceivable that melatonin signals may play an important role in the lunar-age dependent oscillations in expression of the genes for kisspeptin/GnIH system in the grass puffer.

Another possible mechanism could be involved here is the circalunar clock. It has been reported that mRNA levels of cryptochrome (*cry*), one of the circadian clock genes, were higher during a full moon night than a new moon night in the coral (*Acropora millepora*) (Levy et al., 2007). Moreover, lunar phase-dependent expression of *cry* has been reported in the golden rabbitfish (Fukushiro et al., 2011). In this fish, *cry* mRNA levels were peaked at first quarter moon which coincided with spawning. The expression of another circadian clock gene, *period2* was higher during full moon when compared to new moon in the golden rabbitfish (Sugama et al., 2008), supported the possible role of circadian clock genes in the regulation of lunar-synchronized spawning. In the grass puffer, the expression levels of *cry1* and *cry3* showed lunar-age dependent variations in the brain of grass puffer (unpublished data, the 40th Annual Meeting of the Japan Society for Comparative Endocrinology, 2015). Therefore, cryptochrome may also be involved in the regulation of the lunar-age dependent oscillations in expression of the genes for kisspeptin/GnIH system in the grass puffer. The

differential regulatory mechanisms of the kisspeptin/GnIH system regarding the lunar cycle are unknown at present, and needs further investigation.

Table 5.1. Primers used in real-time PCR.

Primers	Nucleotide sequences
tnKiss2-qPCR-F1	5´-GACCTTCAGGGACAACGAGGAC-3´
tnKiss2-qPCR-R1	5´-ATGAAGCGCTTGCCAAAGC-3´
tnKiss2-R-qPCR-F1	5´-TCCCGTTTCTGTTCAAGCACAAG-3´
tnKiss2-R-qPCR-R1	5´-ATTGTTGTTGCGCTCCTCTGC-3´
tnGnIH-qPCR-F1	5′-TGATTCGTCTGTGCGAGGAC-3′
tnGnIH-qPCR-R1	5′-TCAGCAGCTGTGCATTGACC-3′
tnGnIH-R-qPCR-F1	5′-AAGATGCTCATCCTGGTGGC-3′
tnGnIH-R-qPCR-R1	5′-AGATCCACCTGGTCACTGTCC-3′

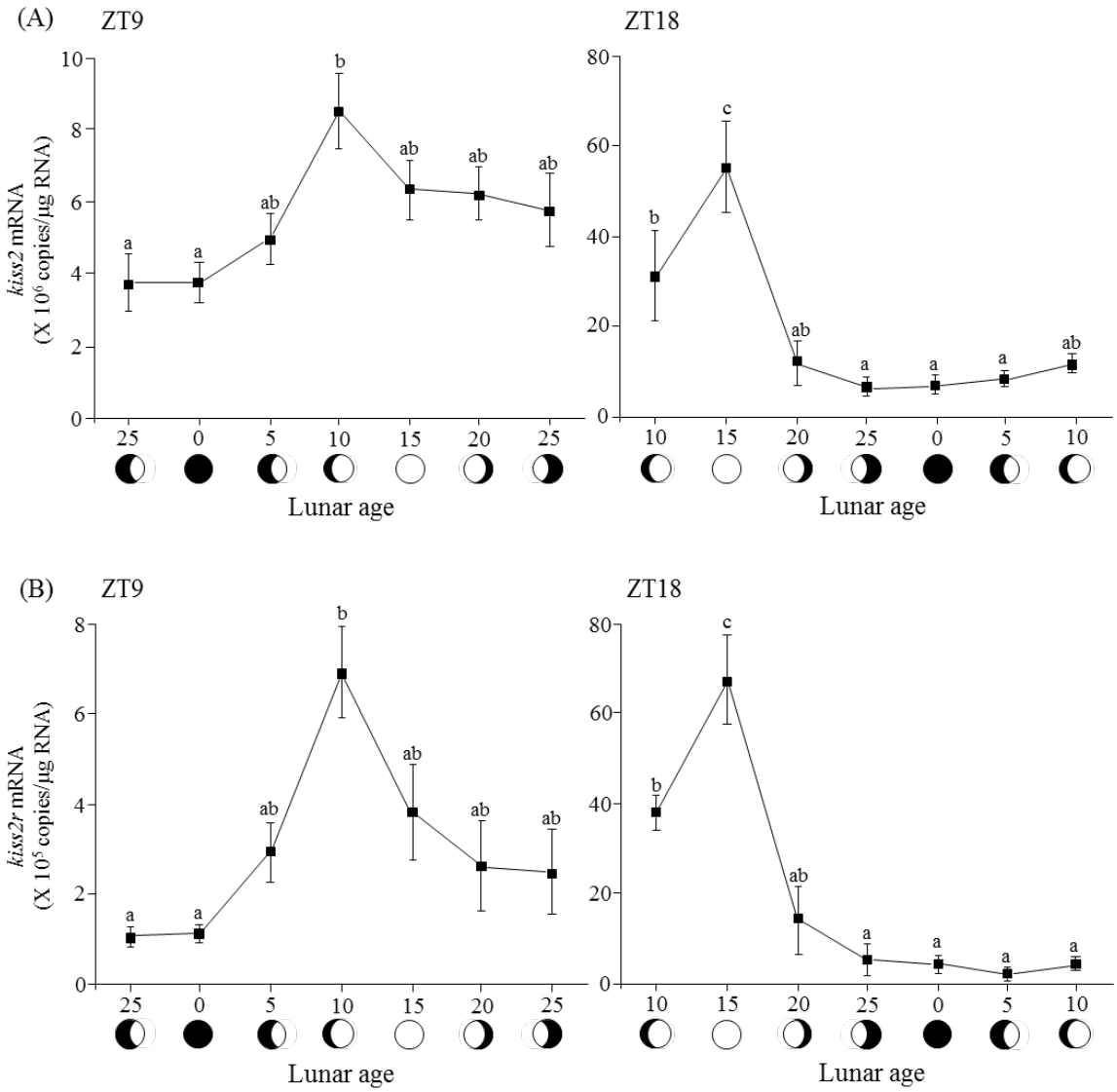

Fig. 5.1. Lunar-age dependent oscillations in expression of *kiss2* (A) and *kiss2r* (B) in the brain. Matured male grass puffer were assessed at different points of a lunar month sampled at ZT9 (daytime) and ZT18 (nighttime) during the breeding season. Values represent the mean ± SEM (n = 8 fish). Values accompanied by different letters are statistically significantly different ($p < 0.05$).

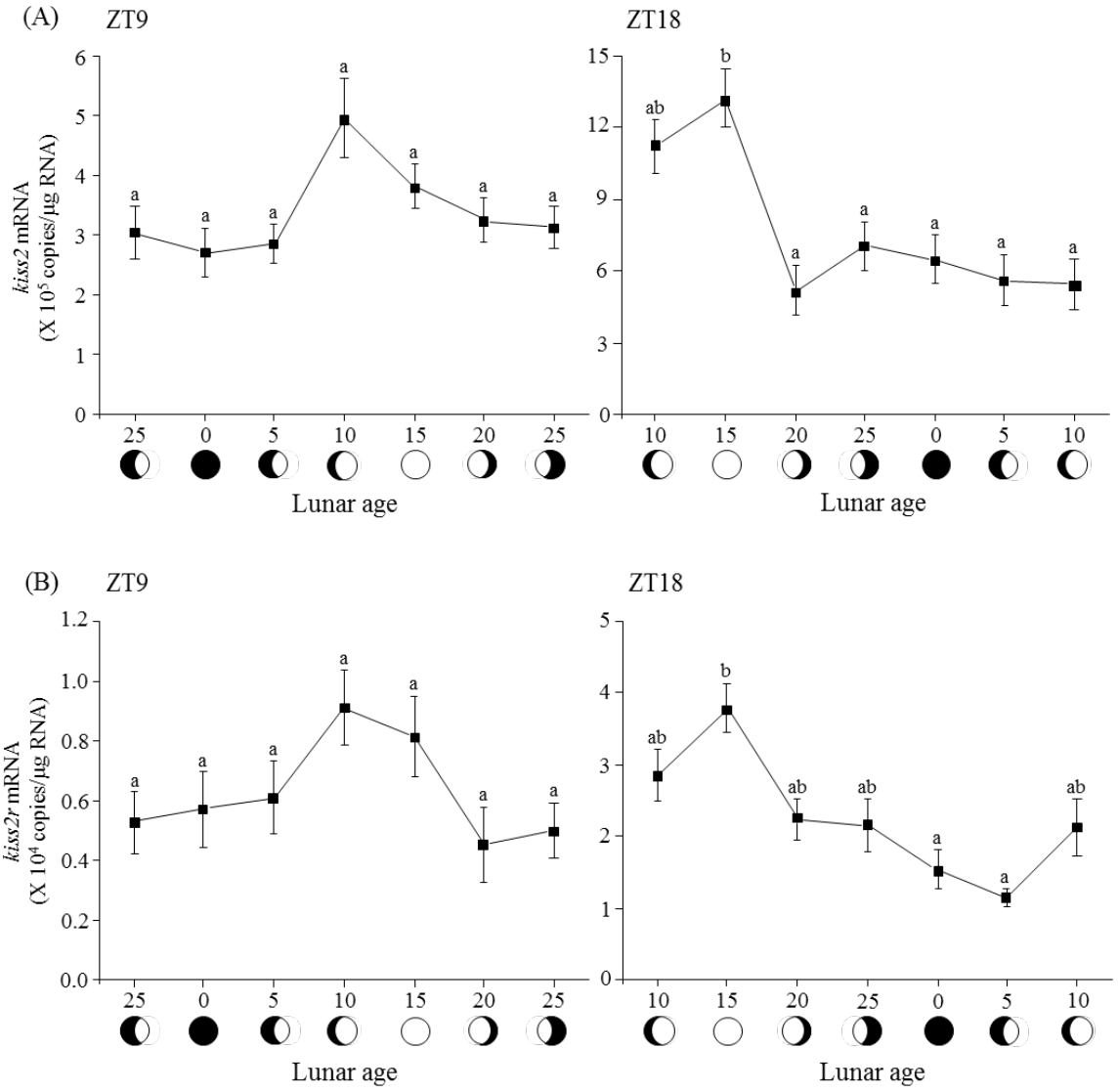

Fig. 5.2. Lunar-age dependent oscillations in expression of *kiss2* (A) and *kiss2r* (B) in the pituitary. Matured male grass puffer were assessed at different points of a lunar month sampled at ZT9 (daytime) and ZT18 (nighttime) during the breeding season. Values represent the mean ± SEM (n = 8 fish/points). Values accompanied by different letters are statistically significantly different ($p < 0.05$).

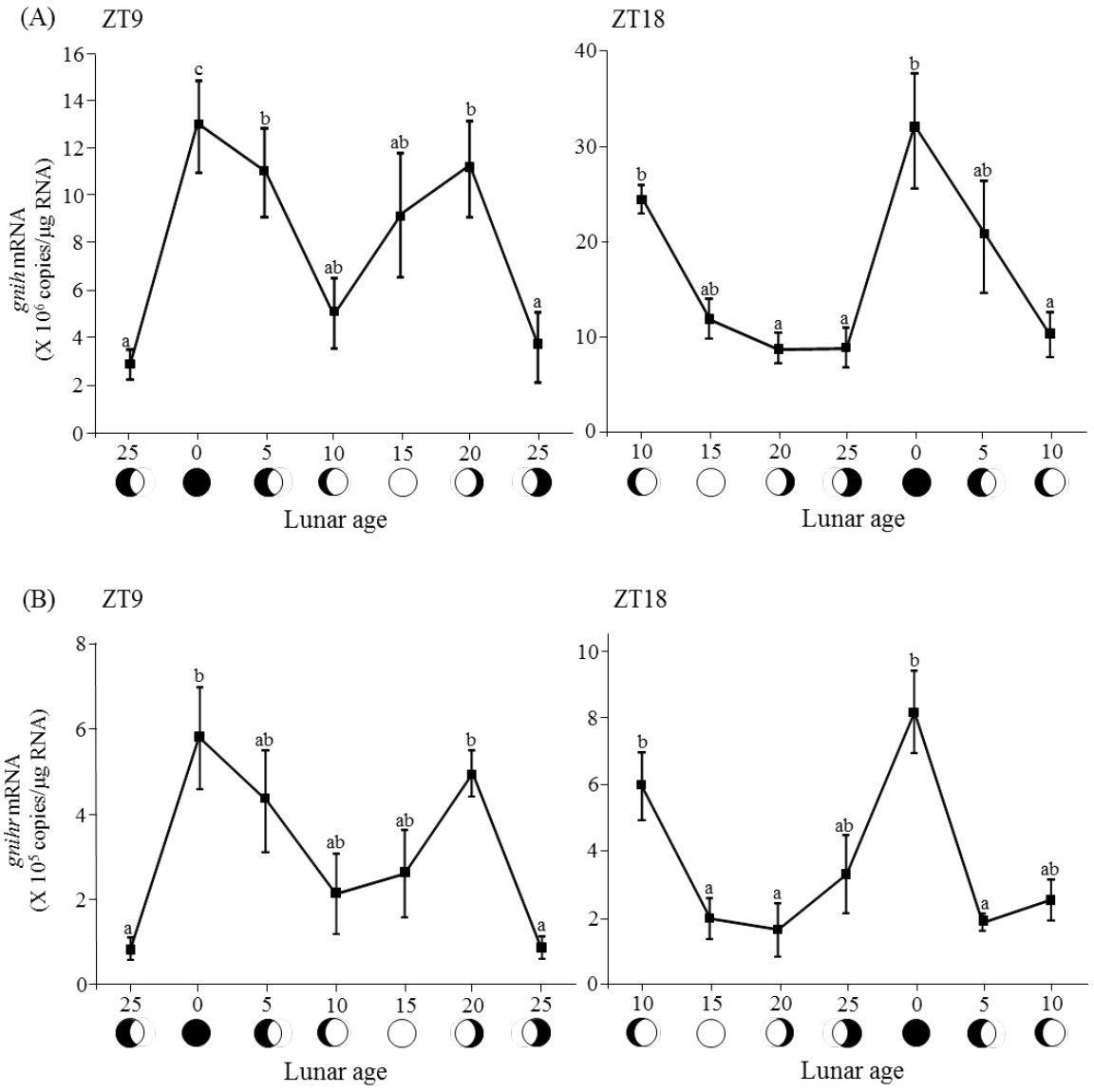

Fig. 5.3. Lunar-age dependent oscillations in expression of *gnih* (A) and *gnihr* (B) in the brain. Matured male grass puffer were assessed at different points of a lunar month sampled at ZT9 (daytime) and ZT18 (nighttime) during the breeding season. Values represent the mean ± SEM (n = 8 fish). Values accompanied by different letters are statistically significantly different ($p < 0.05$).

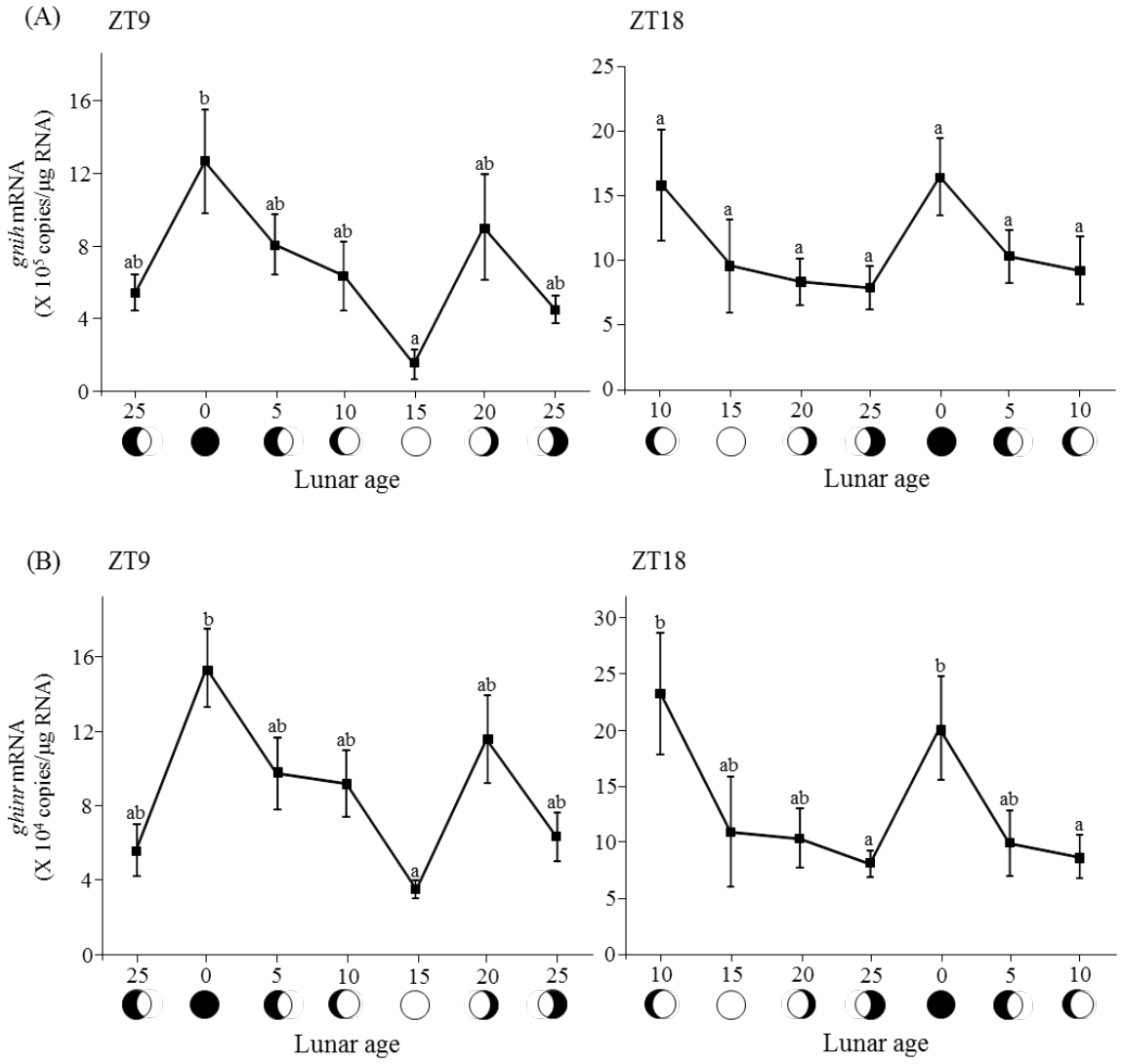

Fig. 5.4. Lunar-age dependent oscillations in expression of *gnih* (A) and *gnihr* (B) in the pituitary. Matured male grass puffer were assessed at different points of a lunar month sampled at ZT9 (daytime) and ZT18 (nighttime) during the breeding season. Values represent the mean ± SEM (n = 8 fish/points). Values accompanied by different letters are statistically significantly different ($p < 0.05$).

Chapter 6

General discussion and conclusions

The present study was conducted to clarify the possible role of the kisspeptin and gonadotropin-inhibitory hormone (GnIH) systems in the regulation of lunar- and tidal cycle-synchronized reproduction of the grass puffer, based on the neuroanatomical studies on kisspeptin/kisspeptin receptor and GnIH/GnIH receptor (GnIH-R) and the changes in expression of these genes in regard to two environmental factors that change periodically during and across the breeding season, i.e. water temperature and lunar cycle. To clarify the role of neuropeptides and their mode of action, the neuroanatomical structure of neurons producing the neuropeptide precursors and their receptors is prerequisite, giving fundamental and useful information to consolidate the complex actions of the multiple neuropeptides involved in the biological phenomenon. Therefore, in the present study, the immunolocalization of Kiss2/Kiss2r and GnIH/GnIH-R was first examined to establish their neuroanatomical structures.

In Chapter 2, Kiss2-ir cells were localized in a single nucleus, the magnocellular preoptic nucleus pars magnocellularis (PMm) in the preoptic area (POA), consistently with the previous study using in situ hybridization (ISH) in the grass puffer (Ando et al., 2014) (Figs 2.1, 2.2A and 2.2C). This was also supported by the findings in other species where *kiss2*-expressing cells were observed in the POA at least as a small group (Kanda et al., 2012; Shimizu et al., 2012; Osugi et al., 2013). Besides, Kiss2r-ir cells could be first detected in the present study, which were localized in three brain regions, the PMm, nucleus dorsomedialis thalami (NDM) and medial preglomerular nucleus (Pgm) (Figs. 2.1, 2.3A-F). This is also consistent with the previous study by Ando et al. (2014) showing the expression of *kiss2r* in the PMm and NDM. The colocalization of Kiss2- and Kiss2r-ir cells in the PMm suggests coexpression of Kiss2 and Kiss2r in single neurons, and there might be autocrine action of Kiss2 in the PMm. Moreover, colocalization of GnIH-ir and GnIH-R-ir cells were demonstrated here in the PMm (Chapter 3, Figs. 3.1D, 3.2A, 3.2B, 3.4D and 3.4E).

Therefore, there might be coexpression of these four molecules in single neurons and/or close proximity of Kiss2 and GnIH neurons in the PMm, suggesting the anatomical and functional interactions of Kiss2 and GnIH neurons.

Furthermore, the PMm is one of the major hypothalamic nuclei that consist of multiple peptidergic neurons, some of which are hypophysiotropic neurosecretory cells including gonadotropin-releasing hormone (GnRH) neurons. Neuroanatomical relations, e.g. coexpression, synaptic contact, cell-cell contact and fiber-fiber contact, between GnRH, kisspeptin and GnIH neurons have been the focus of many studies. In fish, the neuroanatomical and functional relations between them are highly variable depending on fish species and sometimes controversial results have been reported even in the same species possibly due to difference in reproductive stage, season and other environmental conditions. In the grass puffer brain, the colocalization and anatomical relations between GnRH neurons and Kiss2/GnIH neurons and those between Kiss2 and GnIH neurons have not yet been examined, and this should be of considerable interest and importance to further clarify the significant role of the kisspeptin/GnIH system in the regulation of the grass puffer reproduction. Nevertheless, the present study demonstrates the important and interesting neuroanatomical structure of the kisspeptin/GnIH system in the grass puffer.

In Chapter 4, anomalously high temperature (28°C) significantly decreased the levels of *gnih*, *gnihr* and *gh* expressions in the brain and pituitary (Figs. 4.2, 4.3 and 4.4A). Previous study by Shahjahan et al. (2017) showed the suppression effects of the high temperature conditions on the genes for the kisspeptin/GnRH/GTH system. Taking into account that GnIH administration stimulates the expressions of *fshb*, *lhb* and *gh* (Shahjahan et al., 2011; 2016), the present results support that both GnIH and Kiss2 have stimulatory roles in the grass puffer reproduction and the expression of these genes may be important for the maintenance of reproductively active condition. The lunar-age dependent oscillations in expression of *kiss2*, *kiss2r*, *gnih* and *gnihr* during a lunar month revealed in the present study further support this notion (Chapter 5, Figs. 5.1, 5.2, 5.3 and 5.4). The temporal increases in expression of *kiss2* and *kiss2r* at the full moon night and *gnih* and *gnihr* at the new moon are considered to be directly linked to the massive spawning during the spring tide. Although the

lunar-age dependent regulatory mechanisms on these gene expression are currently unknown and will need to be further elucidated, the present results indicate that the periodical changes in the expression and thereby activity of the Kiss2 and GnIH systems are most probably important for the seasonal and lunar-age dependent reproduction of the grass puffer.

In conclusion, the present study reveals the basal and important neuroanatomical structure of the Kiss2 and GnIH systems and the regulation of gene expression in these systems by environmental cues that are connected with seasonal and lunar-age dependent regulation. The present results indicate that Kiss2 and GnIH may play as a key linker for integration of internal and external cues to control the periodic reproduction in the grass puffer. Finally, the present study contributes for understanding the role of the Kiss2/GnIH system in regulating the lunar-synchronized spawning of the grass puffer, and found the following major findings:

1) Kiss2- and Kiss2r-ir cells were localized in the PMm. Kiss2r-ir cells were also distributed in the NDM and Pgm. The number of Kiss2- and Kiss2r-ir cells showed daily variation with higher cell numbers during nighttime.
2) GnIH-ir cells were found in the PMm and TS. GnIH-R-ir cells were widely localized in the telencephalon, diencephalon and mesencephalon including the PMm. Co-localization of Kiss2-, Kiss2r-, GnIH- and GnIH-R-ir cells in the PMm suggests functional interaction between the Kiss2 and GnIH systems to regulate reproduction. GnIH-ir fibers were widely distributed throughout the brain, suggesting neuromodulatory roles of GnIH in non-reproductive functions in addition to reproduction.
3) The expressions of *gnih*, *gnihr* and *gh* were suppressed under the anomalously high temperature conditions along with the suppression of the genes for the kisspeptin/GnRH/GTH system. GnIH and Kiss2 have stimulatory roles in reproduction and their suppression by high water temperature may be important for the termination of breeding season in the grass puffer.
4) The expression of *kiss2*, *kiss2r*, *gnih* and *gnihr* showed lunar-age dependent variations in the brain and pituitary during the breeding season. The expressions of *kiss2* and *kiss2r* were extensively stimulated at the full moon night, whereas the expressions of *gnih* and *gnihr* were significantly activated at the new moon night, suggesting that both Kiss2 and

GnIH play an important role in the semilunar spawning of the grass puffer. Lunar-age dependent mechanisms that differentially regulate the Kiss2 and GnIH systems may be time- and/or light-sensitive in both the brain and pituitary.

To further elucidate the roles of the Kiss2 and GnIH system in the lunar-synchronized reproduction in the grass puffer, the following points will need to be determined: 1) Coexpression and anatomical interactions between Kiss2, GnIH and GnRH neurons; 2) Regulation of expression of the genes for the Kiss2 and GnIH systems by melatonin and circadian clock; 3) Lunar-age dependent molecular mechanisms underlying the regulation of the genes for the Kiss2 and GnIH systems.

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