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Diurnal changes in salmon GnRH secretion in the brain of masu salmon (*Oncorhynchus masou*)

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ABSTRACT

The day–night changes of salmon GnRH (sGnRH), which is secreted from various brain regions, were analyzed in maturing and matured masu salmon (*Oncorhynchus masou*). In maturing males, the levels of sGnRH secreted from the olfactory bulb (OB), terminal nerve (TN), and ventral telencephalon and preoptic area (VT+POA) were all significantly higher during midnight than daytime. However, the contents of sGnRH in the pituitary gland during midnight were not higher than those during daytime. In maturing females, the levels of sGnRH secreted from the VT+POA were higher during midnight than daytime, and the contents of sGnRH in the pituitary gland were also higher during midnight. In matured fish, the levels of sGnRH secreted from the OB, TN and VT+POA during midnight were significantly higher than those during daytime. There were also no significant differences in the contents of sGnRH in the pituitary gland. These results suggest that a short photoperiod may be involved in diurnal secretion rhythms of sGnRH in various brain regions and the pituitary gland.

1. Introduction

Gonadotropin-releasing hormone (GnRH) is considered as one of the most important hormones in gonadal maturation and homing migration in salmonid species (Ueda, 2011). GnRH controls gonadal maturation through the synthesis and release of gonadotropin (GTH) in the pituitary gland (Sherwood, 1987). In salmonid species, gonadal maturation is promoted under a short photoperiod. Moreover, it has been reported that a short photoperiod influences GnRH gene expression in masu salmon (*Oncorhynchus masou*) (Amano et al., 1995). Although GnRH synthesis and secretion are thought to be corresponding to day length (Amano et al., 1994), the actual diurnal rhythms related to GnRH secretions in salmonid species have yet to be clarified.

In salmonid species, two molecular forms of GnRH, salmon GnRH (sGnRH) and chicken-II GnRH (cGnRH-II), are secreted from various brain regions (King and Miller, 1992). sGnRH neurons are distributed widely from the olfactory nerve to the preoptic area (POA) (Amano et al., 1991; Amano et al., 1992). It has been well established that sGnRH systems in the ventral telencephalon (VT) and POA are involved in GTH regulations in the pituitary gland (Amano et al., 1995). Therefore, it has been accepted that the levels of sGnRH secreted from the VT+POA are directly associated with the relative quantity of sGnRH in the pituitary gland. In contrast, it is believed that the wide distributions of sGnRH in various brain regions act as a neuromodulator that is involved in reproductive behavior (Millar, 2003; Oka, 2002). However, in salmonid, there have been not much works focused on sGnRH, which are not involved in GTH regulations. Therefore, further studies of sGnRH not only in VT+POA but also in the other brain regions should be done.

In this study, the diurnal rhythm of sGnRH secretion was assessed in various brain regions in masu salmon using an *in vitro* brain culture and time-resolved fluoroimmunoassay (TR-FIA). The difficulty in collecting secreted sGnRH from the various brain regions in living fish made us to perform the *in vitro* brain culture method. And, TR-FIA is one of the useful method to measure the contents of GnRH forms in another species (Pham et al., 2006; Selvaraj et al., 2012). These experiments were performed using maturing and matured masu salmon brains to investigate diurnal rhythms of sGnRH during maturation. Moreover, the contents of sGnRH in the pituitary gland were also measured by TR-FIA.

2. Materials and methods

2.1. Fish

Masu salmon reared at the Toya Lake Station, Field Science Center for Northern Biosphere, Hokkaido University, were used in

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the present study. Fish were kept in a 2-ton circular tank under a natural photoperiod with a continuous flow of spring water between 9.6 and 10.9 °C. Under natural conditions, masu salmon generally mature in September. Therefore maturing fish were sampled from late June to July (15 light (L):9 dark (D)) and matured fish were sampled in mid-September (12L:12D). The fork length, body weight, gonadosomatic index (GSI) and number of fish are shown in Table 1.

2.2. Sample collection and culture in vitro

Fish were collected during daytime (13:00–14:00 h) and during midnight (01:00–02:00 h) and anesthetized by 0.05% FA-100 (4-allyl-2-methoxyphenol; DS Pharma Animal Health, Osaka, Japan). After decapitation, pituitary glands and brains were collected. Brains were divided into 3 regions (olfactory bulb: OB; terminal nerve: TN; ventral telencephalon and preoptic area: VT+POA) as shown in Fig. 1 referring to the distribution of sGnRH neurons in masu salmon brains (Amano et al., 1991). During midnight, fish were temporally handled, and brains and pituitary glands were collected under weak yellow light. The 3 brain regions were then incubated in 1 ml salmon ACSF (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 5 mM HEPES, pH 7.5; Wako Pure Chemical, Osaka, Japan) (Kitani, 2005) at 15 °C for 2 h under either light or dark environments. The incubation time was determined using sockeye salmon brain (*O. nerka*) (Fig. 2). After incubation, 900 μl of each medium was placed into sample tubes and mixed with 100 μl of 1 N HCl. Each medium solution was lyophilized under a vacuum condition and the pellets were reconstituted in an assay buffer (20 mM sodium phosphate buffer, 0.9% NaCl, 0.1% BSA, 20 μM diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid, 0.01% Tween 40, pH 7.2; all chemicals were purchased from Wako except for BSA was purchased from Sigma–Aldrich, St. Louis, MO, USA). For sGnRH extraction in the pituitary gland, samples were mixed with 1 ml of 0.1 N HCl and homogenized by sonication. After centrifugation at 10,000*g* for 30 min at 4 °C, the supernatant was lyophilized under a vacuum condition and reconstituted in an assay buffer.

2.3. Time-resolved fluoroimmunoassay

To measure the contents of sGnRH in each sample, time-resolved fluoroimmunoassays (TR-FIA) was performed following the method of Yamada et al. Yamada et al. (2002). Fifty microlitre of each extracted sample and biotinylated sGnRH (1 ng/ml) that was prepared following the method of Yamada et al. Yamada et al. (2002) were applied to 96-well microtiter plates (PerkinElmer Finland Oy, Turku, Finland), in which 100 μl of sGnRH antibody that was prepared following the method of Pham et al. Pham et al. (2006) was immobilized by physical adsorption. Following incubation for 18 h at 4 °C, Eu-labeled streptavidin (PerkinElmer Finland Oy) was placed into each well and incubated for 18 h at 4 °C, and then thoroughly washed to remove any unbound Eu-labeled streptavidin. Fluorescence intensity from dissociated Eu was then measured by a microplate reader (Infinite F500, Tecan, Männedorf, Switzerland). The intra- and inter- assay variation of sGnRH were 9.9% and 14.7%. Displacement curve of extracted samples paralleled with the sGnRH standard curves.

Table 1
The information of sex, gonadal maturation stage, fork length (FL), body weight (BW), gonad somatic index (GSI) and number of fish.

Sex	Stage	FL (cm)	BW (g)	GSI (%)	*n*
Male	Maturing	24.6 ± 0.6	258.1 ± 17.7	5.6 ± 0.9	12
	Matured	26.5 ± 0.9	185.6 ± 16.5	0.9 ± 0.1 (spermiated)	18
Female	Maturing	30.0 ± 0.8	162.2 ± 11.7	8.8 ± 0.4	12
	Matured	28.5 ± 0.9	294.1 ± 22.0	0.8 ± 0.1 (ovulated)	18

All values were expressed as mean ± SEM.

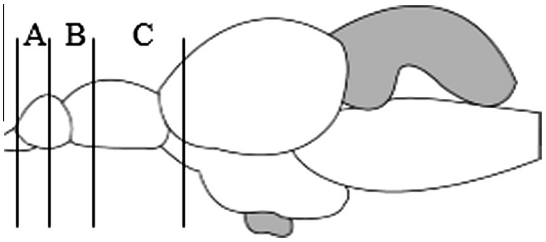

Fig. 1. Divided 3 brain regions for the incubation, OB (A), TN (B) and VT+POA (C).

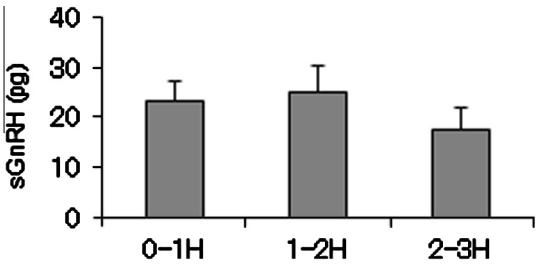

Fig. 2. Secreted sGnRH from VT+POA in various time from the start of incubation, 0–1 h (H), 1–2 h (H) and 2–3 h (H). We used reared sockeye salmon to determine the incubation time. Values are in means ± SEM (n = 5–6).

2.4. Statistical analysis

Values are presented as means ± SEM. Statistical significance was determined using Student's t test and Welch's t test. The differences were considered significant at $p < 0.05$.

2.5. Ethics statement

This study (No. 23-2) has been carried out under the control of the committee following the "Guide for the Care and Use of Laboratory Animals in Field Science Center for Northern Biosphere, Hokkaido University" and Japanese Governmental Law (No. 105) and Notification (No. 6).

3. Results

3.1. Diurnal changes in secretion of sGnRH

In maturing males, the levels of sGnRH secreted from the OB, TN and VT+POA showed significant diurnal changes (Fig. 3A–C). The secreted sGnRH levels during midnight were 3–10 times higher than those during daytime in the OB, TN and VT+POA (Fig. 3A–C). However, in maturing females, significant diurnal changes in the secreted sGnRH levels were only observed in the VT+POA (Fig. 3F). In the OB and TN, the levels of secreted sGnRH tended to be higher during midnight, nevertheless no significant diurnal changes were shown (Fig. 3D and E).

In matured males and females, the levels of sGnRH secreted from the OB, TN and VT+POA showed significant diurnal changes (Fig. 3). During midnight, the secreted sGnRH levels were 2–5 times higher than those during daytime. Out of all sGnRH secretion levels examined, the only secretion that showed significant diurnal changes among both maturing and matured fish of both sexes was sGnRH in the VT+POA (Fig. 3C and F). In general, the secreted sGnRH levels during midnight in maturing fish were higher than those in matured fish.

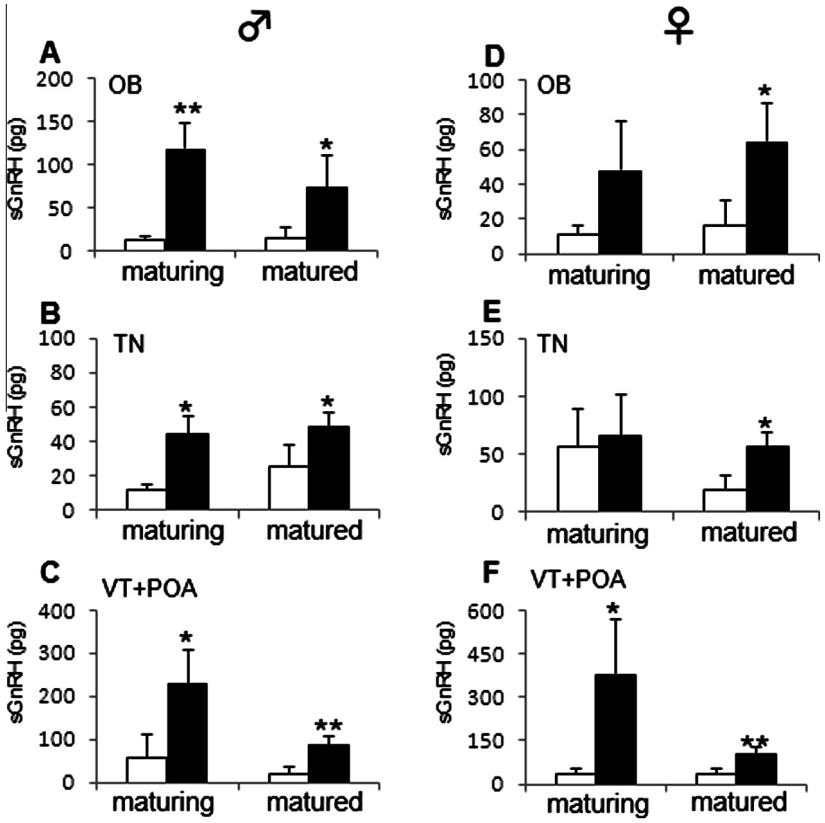

Fig. 3. Diurnal changes in sGnRH secretion from the OB (A and D), TN (B and E) and VT+POA (C and F) in male and female fish. Values are in means ± SEM of males (n = 3–9) and females (n = 4–9) during daytime (open bars) and midnight (shaded bars). $^{**}p < 0.01$ and $^{*}p < 0.05$ indicate statistical significances.

3.2. Diurnal changes in the content of sGnRH in the pituitary gland

The contents of sGnRH in the pituitary gland showed significant diurnal changes in maturing females (Fig. 4B). The contents of sGnRH in the pituitary gland were higher than those during daytime. In maturing males and matured fish, the contents of sGnRH in the pituitary gland showed no diurnal changes (Fig. 4).

4. Discussion

In the present study, the diurnal changes of sGnRH secretions in various brain regions were examined. The levels of sGnRH showed nocturnal elevations with the exception of those secreted from the TN in maturing female. In all cases, the levels of sGnRH secreted from the VT+POA showed significant diurnal changes. It is possible that the activity of sGnRH systems is accelerated under dark environments suggesting that salmonids are short-day breeders.

In medaka (*Oryzias latipes*), medaka GnRH (mdGnRH) neuron firing rates were elevated during the evening (Karigo et al., 2012). mdGnRH neurons are responsible for synthesizing mdGnRH that stimulate the pituitary gland. The luteinizing hormone (LH) surge also occurs during the night in medaka. In salmonids, no clear LH surges were observed (Kobayashi et al., 1987). On the other hand, it is reported that plasma melatonin show diurnal rhythms in masu salmon reared under a long day photoperiod and pre-spawning chum salmon (*O. keta*) (Amano et al., 2000; Saito et al., 2004). Although it is well established that melatonin controls the diurnal regulation of reproduction in many vertebrates, the role of melatonin in fish reproduction has not been clarified (Falcon et al., 2007). Moreover, the diurnal pattern of plasma melatonin is

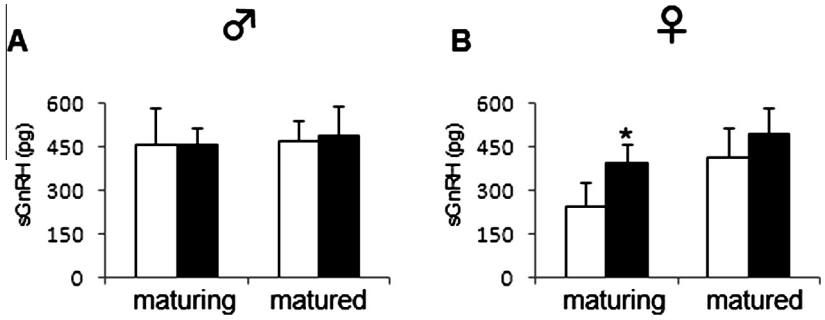

Fig. 4. Diurnal changes in the contents of sGnRH in the pituitary gland in male (A) and female (B) masu salmon. Values are in means ± SEM (n = 3–6) during daytime (open bars) and midnight (shaded bars). $^{*}p < 0.05$ indicates statistical significance.

similar to sGnRH secretion patterns from the VT+POA observed in the present study. Since plasma melatonin undergoes nocturnal elevation, there may be an indirect relationship between melatonin and sGnRH.

During the course of the present study, the characteristics of diurnal secretion patterns of sGnRH in the OB, TN and VT+POA were similar in that sGnRH levels were higher during midnight than daytime. In sockeye salmon, the ontogenic origins of sGnRH neuronal somata were the olfactory placode, and these somata migrated to wide brain regions between the olfactory nerve and preoptic area (Parhar et al., 1995). Therefore, it is considered that control mechanisms of sGnRH neuronal systems may be regulated by same factors.

While sGnRH secreted from the VT+POA showed nocturnal elevation, the contents of sGnRH in the pituitary did not show any clear diurnal changes except in maturing females. In the previous study using masu salmon, short photoperiodic manipulation did not affect the pituitary LH levels (Amano et al., 2001). It is suggested that LH does not have the diurnal rhythm corresponding to the environmental photoperiod. In contrast, we compared the contents of sGnRH in the pituitary gland between the daytime and the midnight by using immature sockeye salmon, and found that clear nocturnal elevations of sGnRH levels in the pituitary gland were observed in both sexes (unpublished data). And in the previous study using chum salmon, it was reported that the diurnal endocrine rhythms may be attenuated in the pre-spawning fish (Saito et al., 2004). From these results, it is considered that the diurnal sGnRH activity in the pituitary gland may not be involved in regulation of the diurnal LH activity and may be attenuated during sexual and gonadal maturation.

In general, it is believed that the ovulating time of salmonid species is not corresponding to the environmental photoperiod. Therefore, the present results suggest that diurnal changes of the VT+POA sGnRH may not be directly involved in reproductive diurnal rhythms. However, we show that sGnRH exhibit diurnal secretion rhythms in both sexual maturing and matured masu salmon. These results conclude that a short photoperiod may be deeply involved in diurnal secretion rhythms of sGnRH in various brain regions.

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