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

Kosuke Fukaya[a], Masafumi Amano[b], Hiroshi Ueda[a,c*]

[a]Division of Biosphere Science, Graduate School of Environmental Science, Hokkaido University, North 9 West 9, Kita-ku, Sapporo, Hokkaido, Japan

[b]School of Marine Biosciences, Kitasato University, Kitasato 1-15-1, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan,

[c]Laboratory of Aquatic Bioresources and Ecosystems, Field Science Center for Northern Biosphere, Hokkaido University, North 9 West 9, Kita-ku, Sapporo, Hokkaido 060-0809, Japan

*Corresponding author: Tel & Fax number: +81-11-706-2598

E-mail address: kosuke@fsc.hokudai.ac.jp (Kosuke Fukaya), amanoma@kitasato-u.ac.jp (Masafumi Amano), hueda@fsc.hokudai.ac.jp (Hiroshi Ueda)

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.

Key words: diurnal secretion, sGnRH, brain, pituitary gland, masu salmon

1. Introduction

Gonadotropin-releasing hormone (GnRH) is considered as one of the most important hormones in gonadal maturation and homing migration in salmonid species [21]. GnRH controls gonadal maturation through the synthesis and release of gonadotropin (GTH) in the pituitary gland [19]. 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*) [4]. Although GnRH synthesis and secretion are thought to be corresponding to day length [3], 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 [10]. sGnRH neurons are distributed widely from the olfactory nerve to the preoptic area (POA) [1, 2]. It has been well established that sGnRH systems in the ventral telencephalon (VT) and POA are involved in GTH regulations in the pituitary gland [4]. 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 [13, 14]. 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 [16, 18]. 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 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 (13:00-14:00h) and during midnight (01:00-02:00h) 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; VT+POA) as shown in Fig. 1 referring to the distribution of sGnRH neurons in masu salmon brains [1]. 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) [11] at 15°C for 2 hours 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 1N HCl. Each

91 medium solution was lyophilized under a vacuum condition and the pellets were
92 reconstituted in an assay buffer (20 mM sodium phosphate buffer, 0.9% NaCl, 0.1%
93 BSA, 20 µM diethylenetriamine-N,N,N',N'',N''-pentaacetic acid, 0.01% Tween 40, pH
94 7.2; all chemicals were purchased from Wako except for BSA was purchased from
95 Sigma-Aldrich, St. Louis, MO, USA). For sGnRH extraction in the pituitary gland,
96 samples were mixed with 1 ml of 0.1N HCl and homogenized by sonication. After
97 centrifugation at 10,000 g for 30 min at 4°C, the supernatant was lyophilized under a
98 vacuum condition and reconstituted in an assay buffer.
99
100 ***2.3. Time-Resolved fluoroimmunoassay***
101 To measure the contents of sGnRH in each sample, TR-FIA was performed
102 following the method of Yamada *et al.* [20]. Fifty µl of each extracted sample and
103 biotinylated sGnRH (1 ng/ml) that was prepared following the method of Yamada *et al*.
104 [20] were applied to 96-well microtiter plates (PerkinElmer Finland Oy, Turku, Finland),
105 in which 100 µl of sGnRH antibody that was prepared following the method of Pham *et*
106 *al*. [16] was immobilized by physical adsorption. Following incubation for 18 hours at
107 4°C, Eu-labeled streptavidin (PerkinElmer Finland Oy) was placed into each well and
108 incubated for 18 hours 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.

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, E).

In matured males and females, the levels of sGnRH secreted from the OB, TN and VT+POA showed significant diurnal changes (Fig. 3A-F). 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, F). In general, the secreted sGnRH levels during midnight in maturing fish were higher than those in matured fish.

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. 4A, B).

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 [9]. 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 [12]. 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*) [6, 17]. 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 [8]. Moreover, the diurnal pattern of plasma melatonin is 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 [15]. 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 [7]. 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 [17]. 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.

5. Acknowledgements

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6. References

[1] M. Amano, Y. Oka, K. Aida, N. Okumoto, S. Kawashima, Y. Hasegawa, Immunocytochemical demonstration of salmon GnRH and chicken GnRH-II in the brain of masu salmon, *Oncorhynchus masou*, J. Comp. Neurol. 314 (1991) 587-597.

[2] M. Amano, K. Aida, N. Okumoto, Y. Hasegawa, Changes in salmon GnRH and chicken GnRH-II contents in the brain and pituitary, and GTH contents in the pituitary in female masu salmon, *Oncorhynchus masou*, from hatching through ovulation, Zool. Sci. 9 (1992) 375-386.

[3] M. Amano, N. Okumoto, S. Kitamura, K. Ikuta, Y. Suzuki, K. Aida, Salmon gonadotropin-releasing hormone and gonadotropin are involved in precocious maturation induced by photoperiod manipulation in underyearling male masu salmon, *Oncorhynchus masou*, Gen. Comp. Endocrinol. 95 (1994) 368-373.

[4] M. Amano, S. Hyodo, S. Kitamura, K. Ikuta, Y. Suzuki, A. Urano, K. Aida, Short photoperiod accelerates preoptic and ventral telencephalic salmon GnRH synthesis and precocious maturation in underyearling male masu salmon, Gen. Comp. Endocrinol. 99

(1995) 22-27.

[5] M. Amano, S. Kitamura, K. Ikuta, Y. Suzuki, K. Aida, Activation of salmon GnRH mRNA expression prior to differentiation of precocious males in masu salmon. Gen. Comp. Endocrinol, 105 (1997) 365-71.

[6] M. Amano, M. Iigo, K. Ikuta, S. Kitamura, H. Yamada, K. Yamamori, Roles of melatonin in gonadal maturation of underyearling precocious male masu salmon, Gen. Comp. Endocrinol. 120 (2000) 190-197.

[7] M. Amano, K. Ikuta, S. Kitamura, K. Aida, Effects of photoperiod on pituitary gonadotropin levels in masu salmon, J. Exp. Zool. 289 (2001) 449-455.

[8] J. Falcon, L. Besseau, S. Sauzet, G. Boeuf, Melatonin effects on the hypothalamo-pituitary axis in fish, Trends. Endocrinol. Metab. 18 (2007) 81-88.

[9] T. Karigo, S. Kanda, A. Takahashi, H. Abe, K. Okubo, Y. Oka, Time-of-day-dependent changes in GnRH1 neuronal activities and gonadotropin mRNA expression in a daily spawning fish, medaka, Endocrinology 153 (2012) 3394-3404.

[10] A. King, R. P. Miller, Evolution of gonadotropin-releasing hormones, Trends Endocrinol. Metab. 3 (1992) 339-346.

[11] T. Kitani, Biochemical and molecular biological study on modulation of gonadotropin-releasing hormone through NMDA receptor in salmonid fish, Master thesis. Hokkaido University (2005).

[12] M. Kobayashi, K. Aida, H. Sakai, T. Kaneko, K. Asahina, I. Hanyu, S. Ishii, Radioimmunoassay for salmon gonadotropin, Nippon Suisan Gakkaishi 53 (1987) 995-1003.

[13] RP. Millar, GnRH II and type II GnRH receptors, Trends Endocrinol. Metab. 14 (2003) 35-43.

[14] Y. Oka, Physiology and release activity of GnRH neurons, Prog. Brain. Res.141, (2002) 259-81.

[15] IS. Parhar, M. Iwata, DW. Pfaff, M. Schwanzel-Fukuda, Embryonic development of gonadotropin-releasing hormone neurons in the sockeye salmon, J. Comp. Neurol. 362 (1995) 256-270.

[16] KX. Pham, M. Amano, N. Amiya, Y. Kurita, K. Yamamori, Distribution of three GnRHs in the brain and pituitary of the wild Japanese flounder *Paralichthys olivaceus*, Fisheries Sci. 72 (2006) 89–94.

[17] D. Saito, Q. Shi, H. Ando, A. Urano, Attenuation of diurnal rhythms in plasma levels of melatonin and cortisol, and hypothalamic contents of vasotocin and isotocin mRNAs in pre-spawning chum salmon, Gen. Comp. Endocrinol. 137 (2004) 62-68.

[18] S. Selvaraj, H. Kitano, M. Amano, H. Ohga, M. Yoneda, A. Yamaguchi, A. Shimizu, M. Matsuyama, Increased expression of kisspeptin and GnRH forms in the brain of scombrid fish during final maturation and ovulation, Reprod. Biol. Endocrinol. 10 (2012).

[19] NM. Sherwood, The GnRH family of peptides, *Trends Neurosci*. 10 (1987) 129-132.

[20] H. Yamada, M. Amano, K. Okuzawa, H. Chiba, M. Iwata, Maturational changes in brain contents of salmon GnRH in rainbow trout as measured by a newly developed time-resolved fluoroimmunoassay, Gen. Comp. Endocrinol. 126 (2002) 136-143.

[21] H. Ueda, Physiological mechanism of homing migration in Pacific salmon from behavioral to molecular biological approaches, Gen. Comp. Endocrinol. 170 (2011) 222-232.

7. Figure captions

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, 0-1hour (H), 1-2 hour (H) and 2-3 (H). We used reared sockeye salmon to determine the incubation time. Values are in means ± SEM (n=5-6).

Fig.3. Diurnal changes in sGnRH secretion from the OB (A, D), TN (B, E) and VT+POA (C, 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.

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.

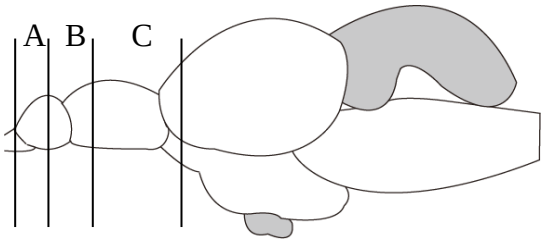

Fig. 1.

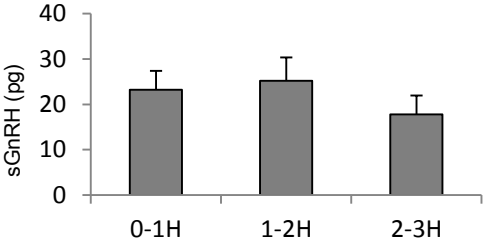

Fig. 2.

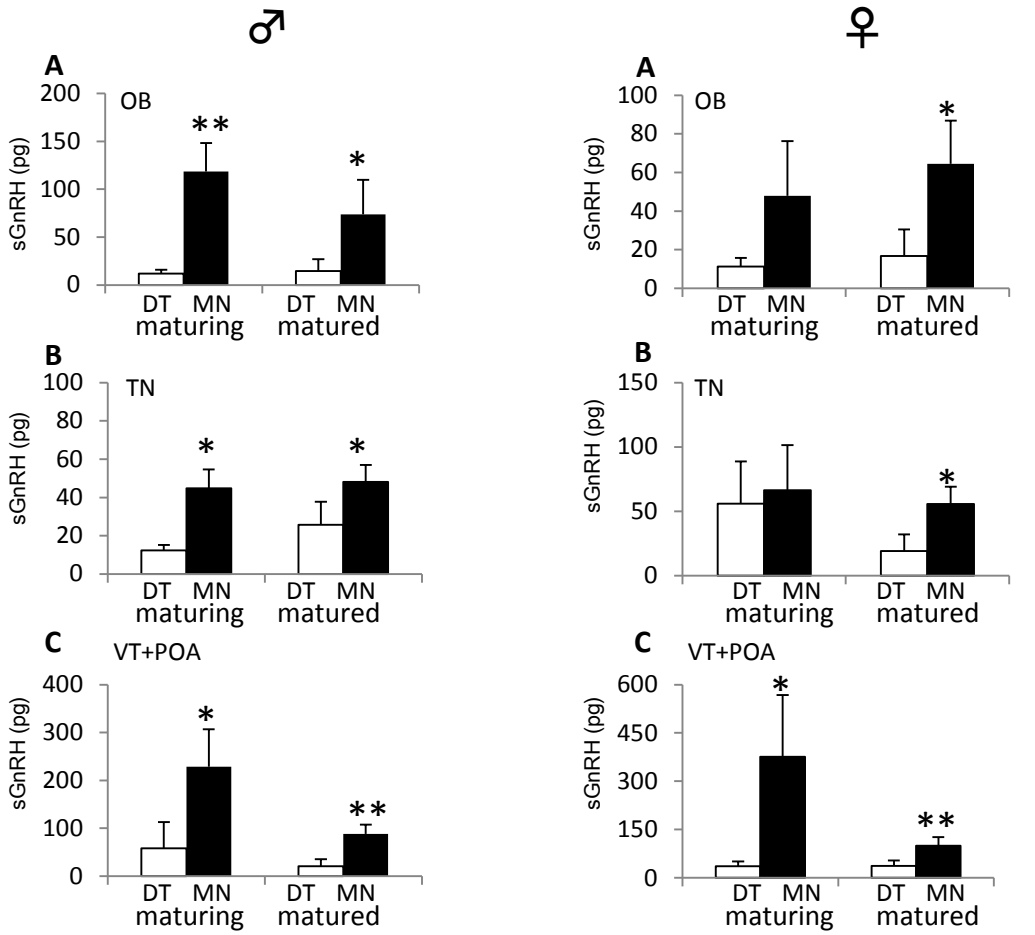

Fig. 3.

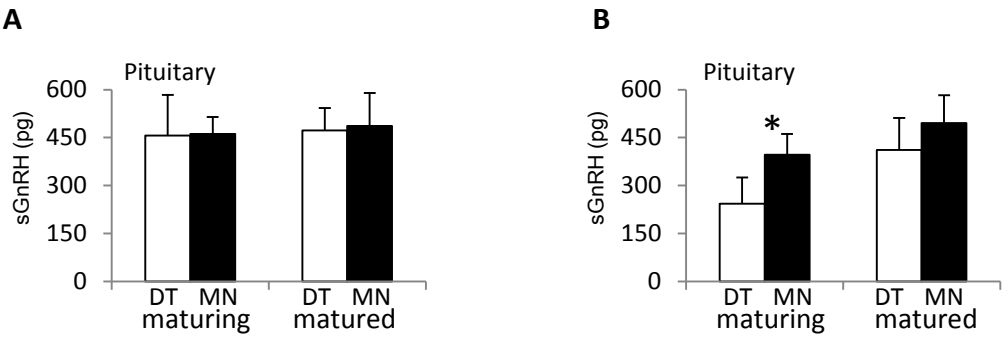

Fig. 5.