

Research paper

Expression profile of GnRH-like peptide during gonadal sex differentiation in the cephalopod kisslip cuttlefish, *Sepia lycidas*Ryosuke Murata^{a,*}, Yuji Mushirobira^a, Yoshiaki Tanaka^b, Kiyoshi Soyano^a^a Institute for East China Sea Research, Organization for Marine Science and Technology, Nagasaki University, Taira-machi, Nagasaki 851-2213, Japan
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ABSTRACT

Gonadotropin-releasing hormone (GnRH) is one of the most important neuroendocrine regulators for animal reproduction. GnRH-like peptide (GnRH-like) has recently been shown to play a critical reproductive role mainly in gametogenesis or steroidogenesis in the gonads of some molluscs, including cephalopods. However, its involvement in gonadal sex differentiation remains unknown. Here, we show the expression profile of GnRH-like in the brain of the cephalopod kisslip cuttlefish, *Sepia lycidas*, throughout gonadal sex differentiation, by quantitative real time RT-PCR and immunohistochemistry. We found that GnRH-like could be detected in the brain at a sexually undifferentiated stage, and its expression level significantly increased upon initiation of gonadal sex differentiation. However, no significant difference in GnRH-like expression levels was observed between sexes during gonadal sex differentiation. Additionally, we demonstrated immunoreactivity of GnRH-like in glial cells or immature neurons, which are mainly distributed in the non-reproductive related area of the cephalopod brain, suggesting the immature function of the reproductive endocrine axis during early ontogenesis. Our results demonstrate for the first time, the expression profile of GnRH-like during early ontogenesis in cephalopods.

1. Introduction

The neuropeptide gonadotropin-releasing hormone (GnRH) plays a central role in vertebrate reproduction (Okubo and Nagahama, 2008). GnRH secreted from neuroendocrine neurons of the hypothalamus typically stimulates the pituitary gland to synthesize gonadotropins, follicle-stimulating hormone, and luteinizing hormone. These pituitary gonadotropins control gonadal synthesis of sex steroid hormones and regulate gametogenesis. It is also well known that GnRH regulates reproductive behavior as a neuromodulator in vertebrates (Okubo and Nagahama, 2008). It has recently been discovered that GnRH-like peptide (GnRH-like) is widely conserved in invertebrates as well, especially in the Mollusca, Annelida, and Echinodermata genera (Roch et al., 2011; Sakai et al., 2017). There are also several reports on the critical involvement of GnRH-like in invertebrate reproduction, especially molluscs, as summarized by Osada and Treen (2013). Thus, GnRH is generally well accepted as one of the most important factors regulating reproduction in a wide range of species. However, comparative and phylogenetic molecular evolutionary analyses has raised questions on the functional homology between vertebrate and invertebrate GnRH (Roch

et al., 2011; Tsai and Zhang, 2008). In fact, it has been reported that GnRH showed little reproductive activity in an in vivo experiment using *Aplysia californica* (Tsai et al., 2010). These facts suggest a functional diversity of GnRH between organisms.

In cephalopods, it has been reported that the endocrine optic glands on the optic tract in the brain (central nervous system, CNS) control the gonadal maturation (Boycott and Young, 1956; Wells and Wells, 1959). For detailed information regarding what each region of the CNS comprises their predicted function, including the optic gland in cephalopods, please refer to previous reports (Amano et al., 2008; Cosmo and Cristo, 1998; Shigeno and Yamamoto, 2002). The complete sequence of brain GnRH-like was first reported in the mollusc cephalopod octopus, as a candidate endocrine factor regulating reproduction (Iwakoshi et al., 2002). It was also suggested that brain GnRH-like may either regulate gonadal maturation or steroidogenesis via an unknown optic gland hormone such as gonadotropin in vertebrates, or directly in the brain through the gonadal endocrine axis, as reported in octopuses (Cosmo and Cristo, 1998; Iwakoshi-Ukena et al., 2004; Kanda et al., 2006). There are only limited studies on reproduction in other cephalopods such as squids and cuttlefish. GnRH-like has also been identified or

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reported in the brains of four squid and cuttlefish species using molecular biological methods or immunohistochemistry (IHC) (Amano et al., 2008; Di Cristo et al., 2009; Onitsuka et al., 2009; Zatylny-Gaudin et al., 2016) (GenBank: KP982885.1). However, to the best of our knowledge, there are no reports to date on the functional reproductive role of GnRH-like in squid or cuttlefish. The expression profile of GnRH-like in the brain with gonadal maturation or differentiation and its relation to the reproductive function of GnRH-like in cephalopods remains unknown.

The kisslip cuttlefish, *Sepia lycidas*, belonging to the Sepiidae family, is widely distributed across the Indian Ocean until the West Pacific Ocean (Natsukari and Tashiro, 1991). The breeding season for kisslip cuttlefish is from spring to early summer, when they exhibit mating behavior and spawning in coastal areas (Natsukari and Tashiro, 1991). Our previous study exploring gonadal sex differentiation in kisslip cuttlefish showed that ovarian differentiation occurs first marked by the appearance of oocytes before hatching, followed by testicular differentiation as detected by the formation of seminiferous tubules, around 20 days post hatching (DPH) (Murata et al., 2019). However, the physiological mechanism of gonadal sex differentiation in cephalopods, particularly in kisslip cuttlefish, remains elusive. Thus, the aim of this study was to clarify the involvement of GnRH-like in gonadal sex differentiation in the kisslip cuttlefish. In this study, we first identified the kisslip cuttlefish *gnrh-like*/GnRH-like (*kc-gnrh*/*kc-GnRH*) from the brain, and analyzed its expression profile throughout gonadal sex differentiation by quantitative RT-PCR and IHC. We also performed dual immunostaining using anti-neurofilament, which is a specific marker for mature neurons, to clarify the *kc-GnRH* immunoreactive (*kc-GnRH-ir*) cell type in the brain of juvenile kisslip cuttlefish. Simultaneously, the distribution of *kc-GnRH* immunoreactivity in peripheral tissues apart from the brain was also investigated by IHC to obtain information on the tissue distribution of cephalopod GnRH-like.

2. Material and methods

2.1. Ethical use of animals

This study was approved by the Animal Care and Use Committee of the Faculty of Fisheries, Nagasaki University (permission no. NF-0043), in accordance with the Guidelines for Animal Experimentation of the Faculty of Fisheries (fish, amphibians, and invertebrates), and by the regulations of the Animal Care and Use Committee, Nagasaki University.

2.2. Animals and sampling procedures

Wild parent kisslip cuttlefish were obtained by line fishing from the shallow coastal area of Nagasaki, or bought from a fish market during their breeding season in 2018 and 2019. Fertilized eggs were obtained from parental fishes, and the embryo and juvenile cuttlefish were raised until 30 DPH, under the same conditions and methods as described by Murata et al. (2019). Before hatching, the eggs were dissected to obtain the cuttlefish embryos. After hatching, prior to dissection, juvenile cuttlefish were anesthetized with 1.0% ethanol in seawater, following the protocol described by Ikeda et al. (2009). The cuttlefishes were sacrificed by decapitation, and the brain and gonads of 30 cuttlefishes were collected at 14, 21, and 28 days after spawning (DAS), and then at 1, 10, 20, and 30 DPH. Whole brains from half of the sacrificed cuttlefishes (15 animals) at each age were fixed in RNAlaterTM Stabilization Solution (Invitrogen, Carlsbad, CA, USA) at 4 °C overnight, and then stored at –30 °C until RNA extraction was performed. The brains from the other half of cuttlefishes (15 animals) and the remaining body of all samples (30 animals) including gonads were fixed in Bouin's solution at room temperature (RT) (15–25 °C) overnight, and transferred to 70% ethanol for storage at 4 °C for normal histology or IHC. The brains of six juvenile cuttlefish at 1 DPH were fixed with 4% paraformaldehyde (PFA) at 4 °C overnight, and then transferred to 70% ethanol for storage at 4 °C for immunofluorescence analysis.

2.3. Histological observation of the gonads throughout sex differentiation

Gonads fixed in Bouin's solution were embedded in paraffin, cross-sectioned, and stained with Delafield's hematoxylin and 1% eosin, using standard methods for light microscopy. All samples were divided into "undifferentiated", "female", and "male" following the histological gonadal status which had been defined by Murata et al. (2019).

2.4. RNA extraction and cloning of *kc-gnrh*

Total RNA was extracted from the brain samples using the ISOGEN II (Nippon Gene, Tokyo, Japan) following the manufacturer's protocols. RNA concentration was measured using a NanoDrop 2000 Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) and used further for cloning and quantitative RT-PCR.

One microgram total RNA was reverse-transcribed using an oligo (dT) primer and Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. PCR was performed using degenerate primers (forward: 5-taccaytttagcaatgatggcac, reverse: 5-aktttytckatcaaagcyttgtt) designed from a highly conserved region of *gnrh-like* of three cephalopod species (*Octopus vulgaris*: AB037165.1, *Uroteuthis edulis*: AB447557.1, and *Sepiella japonica*: KP982885.1). Tks Gflex DNA polymerase (Takara Bio, Kusatsu, Japan) was used according to the manufacturer's instructions. Amplified products were separated by 2.0% agarose gel electrophoresis and purified from excised gel fragments using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The isolated sample was ligated with a plasmid vector using a TOPO TA Cloning Kit (Invitrogen) after adding 3' A-overhangs, and the transformants containing the recombinant plasmid were generated with Competent Quick DH5α cells (Toyobo, Osaka, Japan). Recombinant clones selected by blue-white screening were then used to extract and purify plasmid DNA using the NucleoSpin Plasmid EasyPure kit (MARCHEREY-NAGEL, Düren, Germany), and the purified plasmid was sequenced by FASMAC (Atsugi, Japan). Based on the sequence of the presumptive *kc-gnrh*, Rapid amplification of cDNA ends (RACE) was performed to isolate the 5' and 3' ends of the cDNA using SMARTer RACE 5'/3' kit (Takara Bio) according to the manufacturer's instructions. The mRNA used for RACE was purified from the total RNA using an Oligotex-dT30 < Super > mRNA Purification Kit (Takara Bio). PrimeSTAR Max DNA Polymerase (Takara Bio) was used for RACE PCR. Gene-specific primers (GSPs) in combination with the vector sequence at the 5'-end (5'-RACE: 5'-gattacgccaagcttggaaatcacttcgttaccaccaag, 3'-RACE: 5'-gattacgccaagctgcacctgggtttaac-gaagtggac) and a Universal Primer A Mix, were used for primary PCR. In the 5' RACE procedure, nested PCR was conducted using nested GSP (5'-gattacgccaagctccaccagggtccatcattgctaa) and a Universal Primer Short after the primary PCR.

Sequences were analyzed and aligned using the Basic Local Alignment Search Tool (BLAST; <https://www.ncbi.nlm.nih.gov>) and Multiple Sequence Comparison by Log-Expectation Tool (MUSCLE; <https://www.ebi.ac.uk>). The functional domain of *kc-GnRH* was deduced from conserved domains in the other octopus and squid GnRH sequences (Minakata and Tsutsui, 2016).

2.5. Quantification of *kc-gnrh*

kc-gnrh in the brain was quantified using real-time quantitative-PCR. Brain samples (5 to 7 each) from each sexual stage and at each age were reverse-transcribed from 500 ng of total RNA in a 10 μL reaction volume, using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo) according to the manufacturer's instructions. The copy number of *kc-gnrh* was estimated based on the plasmid standard. The primer pair (forward: 5-ggcctttcacctgtcta, reverse: 5-tgtctggaaatccacttcgtt, amplicon size: 75 bp) was designed using Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>). The dilution templates (corresponding to 50 ng total RNA/well) for each sample were

tested in 10 µL of PCR mixture containing 2 × KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA) and 1 µM of each primer. The PCR protocol was as follows: 1 cycle of initial degeneration at 95 °C for 3 min, 40 cycles of degeneration at 95 °C for 10 s, annealing at 60 °C for 20 s, and extension at 72 °C for 1 s, followed by melting curve analysis. Non-specific amplifications were detected using melting curve analysis. Both PCR amplification and fluorescent detection were performed using a LightCycler 480 (Roche). One-way analysis of variance (ANOVA) followed by the Tukey–Kramer comparison test was used for statistical analysis.

2.6. Antibody, immunohistochemistry, and immunofluorescence

An oligo-peptide corresponding to the partial kc-GnRH amino acid sequence NYHFSNGWHPGGKRSGLPDMQC, was used to generate a specific antibody. Japanese white rabbits (specific pathogen-free animal; SPF) were immunized with the synthesized oligo-peptide (four times, once every two weeks), this procedure was performed by Cosmo Bio (Tokyo, Japan).

The IHC method used to investigate the expression of kc-GnRH in the brain or peripheral tissues is as described by Murata et al. (2011). Brain samples from each sexual stage (5 to 7 each) at each age were used for IHC. Briefly, 5 µm tissue sections fixed in Bouin's solution were deparaffinized with xylene, rehydrated in graded alcohols, and finally washed with phosphate-buffered saline (PBS). The sections were then treated with 3% H₂O₂/methanol for 15 min to inactivate endogenous peroxidase activity, incubated with 10% normal goat serum for 15 min to eliminate non-specific binding, and incubated overnight with the primary antibody (diluted 1:4000 in 1% BSA/PBS) in a moist chamber at 4 °C. The primary antibody against kc-GnRH (anti-kc-GnRH) was detected and visualized using the Histofine anti-rabbit IHC kit and diaminobenzidine (Nichirei, Tokyo, Japan), in accordance with the manufacturer's protocol. The histological part of the brain used for IHC was determined as described in Amano et al. (2008). The adjacent sections, considered as control sections, were incubated in the following conditions: original antigen-adsorbed primary antibody (diluted 1:4000 in 1% BSA/PBS, following adsorption with 400-folds of the weight of antigen), solvent alone (1% BSA/PBS), and pre-immunized serum from the rabbit used for antibody generation (diluted 1:4000 in 1% BSA/PBS). No significant background immunoreactive signals were detected in the control group (Supplementary Fig. 1).

Brain samples fixed with 4% PFA for immunofluorescence were sectioned at 5 µm intervals using the same methods as for IHC analysis. In addition to anti-kc-GnRH (host species: rabbit), the primary monoclonal antibody against neurofilament M/H (anti-NF-M/H, BioLegend, San Diego, CA, USA, host species: mouse) was used for double immunofluorescence. The immunoreactive specificity of anti-NF-M/H for squids was confirmed by the manufacturer's test. The brain sections were deparaffinized, washed in PBS, and the slides incubated with 5% normal goat serum in PBS/0.3% Triton X-100 for 1 h at RT to eliminate non-specific binding. The slides were then immediately incubated at 4 °C overnight with a mixture of the primary antibodies: anti-kc-GnRH and anti-NF-M/H (diluted 1:1000 for anti-kc-GnRH, and 1:2000 for anti-NF-M/H, in 1% BSA/PBS/0.3% Triton X-100). The slides were washed three times (5 min for each wash) with PBS/0.1% Tween 20 (PBST), and then incubated at RT for 60 min in the dark with a mixture of the secondary antibodies: Alexa Fluor 488 goat anti-rabbit IgG, and Alexa Fluor 594 goat anti-mouse IgG (diluted 1:1000 in 1% BSA/PBS/0.3% Triton X-100). The slides were then washed three times with PBST (5 min for each wash) and mounted in glycerin containing DAPI (Pro-Long Diamond Antifade Mount with DAPI; Invitrogen). The slides were dried at RT overnight in the dark, followed by fluorescence microscopy analysis (BZ-X710; Keyence, Osaka, Japan).

3. Results

3.1. Morphological characteristics of gonadal sex differentiation

Embryo cuttlefish at 14–21 DAS had an undifferentiated gonad consisting of somatic cells and germ cells (Fig. 1a). Ovarian differentiation first showed the appearance of meiotic oocytes in the gonads of female cuttlefish at around 28 DAS, followed by testicular differentiation in the male gonad, evident from the formation of seminiferous tubules at around 20 DPH, as reported by Murata et al. (2019) (Fig. 1b and c).

3.2. Cloning of *kc-gnrh*

Partial sequences of 101 bp were obtained after amplification of the first strand cDNA from the kisslip cuttlefish brain, using degenerate primers. Homology analysis showed this fragment to be cephalopod *gnrh-like*. RACE reactions using specific primers resulted in isolating the overlapping 5'- and 3'- sequences for a total length of 723 bp (GenBank accession no. LC550284). The GnRH-like protein sequence as deduced from its gene sequence, was 90 amino acids long and contained conserved functional domains present in GnRH-like of other cephalopods (Fig. 2).

3.3. Quantification of *kc-gnrh* in the brain throughout gonadal sex differentiation

kc-gnrh was detected in the brain at the undifferentiated stage (14 to 21 DAS), and it rapidly increased at the initiation time of morphological ovarian differentiation in females (28 DAS) (Fig. 3). Subsequently, the *kc-gnrh* level was slightly decreased, and this level was maintained until 30 DPH. No significant difference was observed in the expression level of *kc-gnrh* in the brain between the sexual stages throughout gonadal sex differentiation.

3.4. Localization of kc-GnRH immunoreactive cells in brain throughout gonadal sex differentiation

The kc-GnRH-ir cell bodies were observed in the palliovisceral lobe (pvL) and ventral magnocellular lobe (vML) of the brain at the undifferentiated stage (14 DAS) (Fig. 4 a–d). The kc-GnRH-ir cell bodies and fibers in the same area tended to increase upon ovarian differentiation (28 DAS), and were also observed after hatching (Fig. 4 f–i and k–n). Although a few kc-GnRH-ir fibers were seen in other areas of the brain, including the optic gland, there were no kc-GnRH-ir cell bodies observed (Fig. 4 e, j, and o). No sexual stage-based differences were observed in the distribution of kc-GnRH-ir cell bodies and fibers in the brain throughout gonadal sex differentiation.

Dual immunofluorescence showed that kc-GnRH-ir cell bodies were not co-localized with immunoreactivity against anti-NF-M/H in the brain of juvenile kisslip cuttlefish at 1 DPH (Fig. 5). The kc-GnRH-ir cells exhibited small shapes. On the other hand, the NF-M/H-immunoreactive cells were larger in shape with clear axons (Fig. 5).

3.5. Distribution of kc-GnRH immunoreactivity in the peripheral tissues of juvenile kisslip cuttlefish

The kc-GnRH-ir fibers were observed in the luminal side of the heart auricle of juvenile kisslip cuttlefish at 10 DPH (Supplementary Fig. 2). No kc-GnRH-ir cell bodies or fibers were observed in peripheral tissues other than the heart at this stage.

4. Discussion

To understand the involvement of the brain GnRH-like in gonadal sex differentiation, we cloned *gnrh-like* from kisslip cuttlefish. Our results

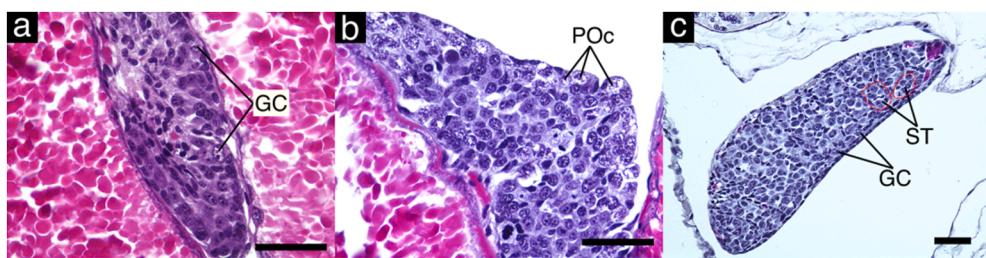


Fig. 1. Gonadal sections of kisslip cuttlefish at the undifferentiated stage (21 DAS; a), the initial stage of ovarian differentiation (28 DAS; b), and testicular differentiation (20 DPH; c). The red dashed line in the figure (c) indicates the seminiferous tubule. DPH, days post hatching; GC, germ cell; POC, primary oocyte; ST, seminiferous tubule. Scale bars = 50 μ m.

<i>Octopus vulgaris</i>	MSATASTTSSRKMAFFIFSMILLSLCLQTOAQNYHFSNGWHPGGKRSALSIDIQCHFRQTKALIEKILDEEINRIIITCTGPVNEIADL—	89
<i>Uroteuthis edulis</i>	MSTSPVSTLRRMVFLTCIAIFLLSLCMQTOAQNYHFSNGWHPGGKRSGIPDMQCHFRPQTKALIEKILDEEIRIITLTCTNTVNIDADLQ	90
<i>Sepia lycidas</i>	MSTSTASSSLRMAFFTCAILLLSFCMHIAQNYHFSNGWHPGGKRSGLPDMQCHFRPQTKALIEKILDEEIRIITLTCTNTVNIDADLQ	90
<i>Sepiella japonica</i>	MSTTALSSNLRKMAFLTCAIPLLSCFMQIQAQNYHFSNGWHPGGKRSGLPDMQCHFRPQTKALIEKILDEEIRIITLTCTNTVNIDADLQ	90
<i>Sepia officinalis</i>	MSTSALSSNLRKMAFLTCAIPLLSCFMQIQAQNYHFSNGWHPGGKRSGLPDMQCHFRPQTKALIEKILDEEIRIITLTCTNTVNIDADLQ	90
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Fig. 2. Alignment of precursors of cephalopod GnRH-like peptides. Shaded regions are the conserved functional regions of GnRH.

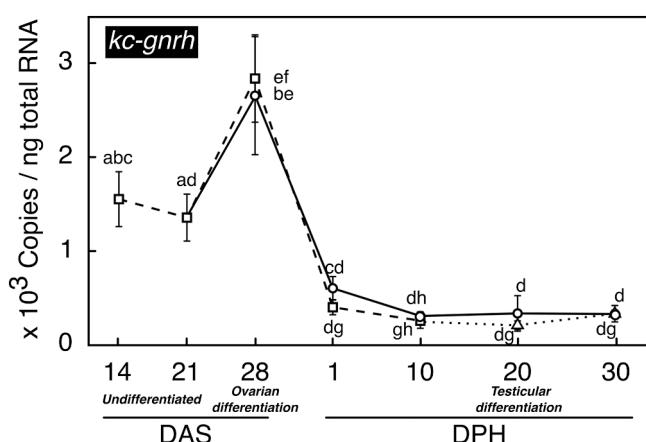


Fig. 3. The expression profile of *kc-gnrh* gene in the brain of kisslip cuttlefish throughout gonadal sex differentiation period. Square marker and dashed line indicate undifferentiated stage, circle marker and solid line indicate female, and triangle marker and dotted line indicate male. DAS, days after spawning; DPH, days post hatching. The different letters indicate statistical differences among sex and stages (two-way factorial ANOVA followed by Tukey's HSD test, $p < 0.05$).

clearly indicate that the functional region of GnRH-like is highly conserved among cephalopods, as reported by Minakata and Tsutsui (2016). Our present study is the fourth report of *gnrh-like* cDNA cloning in squid or cuttlefish, and the fifth in cephalopods. To clarify the involvement of GnRH-like in gonadal sex differentiation, we investigated the expression profile of *kc-gnrh*/GnRH in the brain during this process. Our results clearly demonstrated that *kc-gnrh*/GnRH was expressed in the brain of the embryo or juvenile kisslip cuttlefish at the sexually undifferentiated stage itself, suggesting that *kc-GnRH* already has a functional role at this stage. Our results also demonstrated that *kc-GnRH*-ir fibers were seen in the heart of juvenile kisslip cuttlefish, suggesting not only a modulatory function in heart contractions, but also a multifunctional role of GnRH-like, similar to that reported in octopuses (Iwakoshi-Ukena et al., 2004). This is the first report revealing the expression profile of GnRH-like during early ontogenesis in cephalopods.

In the well-studied vertebrate teleost fish, gonadal sex differentiation is mainly triggered by a sex steroid hormone which is secreted from the gonads, with limited involvement of the pituitary gonadotropins or

brain GnRH (Yan et al., 2012). On the other hand, it has been reported that GnRH-like directly stimulates gonadal gametogenesis in the molluscs scallop *Patinopecten yessoensis*, and in the abalone *Haliotis asinina*, suggesting its direct involvement in gonadal sex differentiation (Nagasaki et al., 2015; Nakamura et al., 2007; Nuurai et al., 2016). In the present study, we demonstrated that active GnRH-like is expressed in both the sexually undifferentiated kisslip cuttlefish brain as well as during gonadal sex differentiation. However, no sexual difference was observed in the expression level of *kc-gnrh* and the appearance or distribution of *kc-GnRH* immunoreactivity in the brain throughout sex differentiation. A definitive sexual dimorphism is observed in the expression profile of factors critical for gonadal sex differentiation in teleost fishes (Jiri et al., 2008). Therefore, we propose that brain GnRH-like is unlikely to be a direct trigger of gonadal sex differentiation in the kisslip cuttlefish.

Our results also demonstrate that the expression level of *kc-gnrh* in the brains of both presumptive male and female kisslip cuttlefish was significantly increased at the initiation time of morphological ovarian differentiation in females. Consequently, the number of *kc-GnRH*-ir cell bodies also increased, suggesting the activation of GnRH-like in both sexes. GnRH-like has functional roles other than reproduction in octopuses such as, autonomic function, feeding, memory, and movement, as revealed by the expression analysis of the GnRH-like receptor (Kanda et al., 2006). Our previous study and the present study collectively conclude that the ovarian differentiation period, shows temporary activation of brain *kc-GnRH* in both sexes, and that this occurs before hatching, which is the starting period for feeding or moving during early ontogenesis (Murata et al., 2019). From these facts, we assume that the activated *kc-GnRH* during the ovarian differentiation period may have some functional role, not on gonadal differentiation, but on the initiation of feeding or moving. Further analysis of the GnRH-like receptor is required to clarify the involvement of GnRH-like as a direct regulating factor of early ontogenesis, including gonadal sex differentiation in kisslip cuttlefish.

It has already been proved by the surgical removal experiment that gonadal maturation in the cephalopod octopus is neurally controlled by the optic gland, which is considered to be a neuroendocrine organ in the brain, as summarized by Cosmo and Cristo (1998). Subsequently, GnRH-like immunoreactivity was demonstrated in the regulating area of the optic gland, suggesting the critical role of GnRH-like as an upstream regulator of reproduction in octopus (Cosmo and Cristo, 1998). In the adult spear squid, GnRH-like immunoreactivity was also detected around the optic gland area, as well as in the pVL and vML, giving rise to

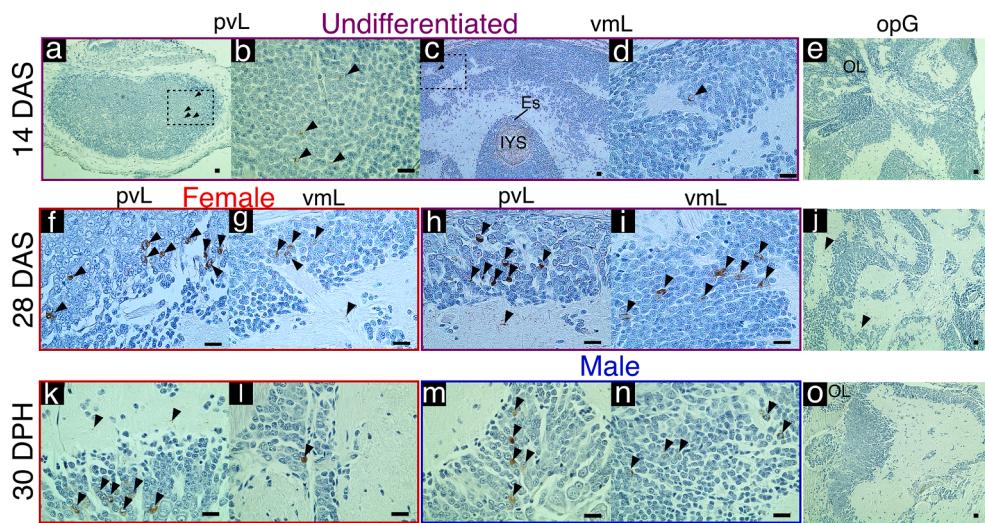


Fig. 4. Representative cross sections of kisslip cuttlefish brains immunostained with anti-kc-GnRH throughout gonadal sex differentiation period. Arrowheads indicate GnRH immunoreactive cell bodies or fibers. The cross sections of palliovisceral lobe (pvL) (a and b), ventral magnocellular lobe vmL (c and d), and optic gland (opG) (e) at undifferentiated stage (14 DAS). b and d are magnified images of the dashed line boxed areas in a and c, respectively. The cross sections of female pvL and vmL (f and g), undifferentiated stage pvL and vmL (h and i), and female opG (j) at 28 DAS, respectively. The cross sections of female pvL and vmL (k and l), male pvL and vmL (m and n), and female opG (o) at 30 DPH, respectively. DAS, days after spawning; DPH, days post hatching; Es, esophagus; IYS, internal yolk sac; OL, optic lobe; opG, optic gland; pvL, palliovisceral lobe; vmL, ventral magnocellular lobe. Scale bars = 20 μ m.

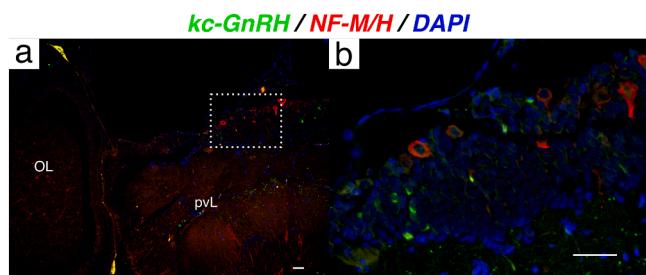


Fig. 5. Cross section of palliovisceral lobe (pvL) area of the 1 DPH kisslip cuttlefish brain immunostained with anti-kc-GnRH (green) and anti-neurofilament-M/H (red). Nuclei were visualized with DAPI staining (blue). (b) is a magnified image of the dashed line boxed area in (a). DPH, days post hatching; NF-M/H, neurofilament-M/H; OL, optic lobe; pvL, palliovisceral lobe. Bars = 50 μ m.

the assumption that GnRH-like may regulate reproduction through the optic gland (Amano et al., 2008). Thus, in the case of adult cephalopods, GnRH-like usually shows active immunoreactivity around the optic gland suggesting a role in regulating reproduction. However, the present study is the first to demonstrate the location of kc-GnRH-ir cell bodies only in the pvL and vml in the brain of kisslip cuttlefish at an early developmental stage, and not in the optic gland area. A morphological study of the pygmy cuttlefish, *Idiosepius paradoxus*, summarized that the major function of pvL and vml in the brain is presumably not in reproduction, but muscle control, ink emission, or jet propulsion (Shigeno and Yamamoto, 2002). In addition, our present study demonstrates for the first time that kc-GnRH-ir cell bodies in the brain are not colocalized with neurofilaments, which is the specific structure for mature neurons, including neuroendocrine neurons (Lee et al., 1988; 1987). This result indicates that kc-GnRH is distributed in glial cells or immature neurons and mainly plays a supporting role for functional neurons during early ontogenesis (Freeman, 2015). In the case of adult octopus or squid, GnRH-like immunoreactivity was observed in neurons with active fibers in the brain, regulating reproduction as a neuroendocrine factor (Amano et al., 2008; Cosmo and Cristo, 1998; Di Cristo et al., 2009). These observations indicate that the function of the endocrine axis consisting of the optic gland and gonad, might control reproduction in cephalopods, might be immature in the early developmental stages of the kisslip cuttlefish. Consequently, it is unlikely that brain kc-GnRH is involved as an upstream factor in gonadal sex differentiation. There is limited definitive knowledge regarding the function of each part of the

brain as well as the endocrine axis from the optic gland to the gonad, regulating reproduction in squid or cuttlefish; therefore, further studies are needed to conclusively define the involvement of GnRH-like in sex differentiation.

Molecular evolutionary and phylogenetic studies on vertebrate and invertebrate GnRH recently revealed that GnRH and GnRH-like belong to a larger peptide family consisting of GnRH, adipokinetic hormone, corazonin, and adipokinetic hormone/corazonin-related peptides, and that these peptides might share a common ancestor (Lindemans et al., 2010; Tsai, 2018; Tsai and Zhang, 2008; Zandawala et al., 2018). Additionally, several reports have indicated a functional diversity of GnRH and GnRH-like with the evolution of not only reproductive roles but also cardiac regulatory functions (Iwakoshi-Ukena et al., 2004; Mitsuhashi et al., 1999; Tsai et al., 2010). Our present study also suggests that kc-GnRH has some involvement in the development of peripheral organs other than the gonads, such as the heart or muscle, during early ontogenesis in kisslip cuttlefish. From these facts, we suggest the possibility that GnRH-like might have little effect on the reproductive activity in the kisslip cuttlefish. Further functional studies of GnRH on reproductive activity will be needed to elucidate this prediction.

In conclusion, this study reveals, for the first time, the expression profile of GnRH-like in the brain during the early development stage of kisslip cuttlefish throughout gonadal sex differentiation. We have successfully demonstrated that no sexual difference was observed in the expression of GnRH-like in the brain during early ontogenesis. These findings provide fundamental insights into not only the physiology of cephalopod reproduction, but also the functional diversity of GnRH between organisms.

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CRediT authorship contribution statement

Ryosuke Murata: Conceptualization, Methodology, Investigation, Investigation, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition. **Yuji Mushirobira:** Methodology, Investigation, Writing - review & editing. **Yoshiaki Tanaka:** Resources, Writing - review & editing. **Kiyoshi Soyano:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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