Highlights

- Kisslip cuttlefish *gnrh-like* was cloned as the 5th report in cephalopods
- *gnrh-like* rapidly increased in the brain at the ovarian differentiation
- GnRH-like was detected in the brain throughout gonadal sex differentiation
- No sex difference was seen in GnRH-like levels during gonadal sex differentiation

Title: Expression profile of GnRH-like peptide during gonadal sex differentiation in the cephalopod kisslip cuttlefish, *Sepia lycidas*

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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.

Keywords: Gonadotropin-releasing hormone, mollusc, reproduction, sex, squid

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

46 regulating reproduction in a wide range of species. However, comparative and phylogenic
47 molecular evolutionary analyses has raised questions on the functional homology
48 between vertebrate and invertebrate GnRH (Roch et al., 2011; Tsai and Zhang, 2008). In
49 fact, it has been reported that GnRH showed little reproductive activity in an in vivo
50 experiment using *Aplysia californica* (Tsai et al., 2010). These facts suggest a functional
51 diversity of GnRH between organisms.

52 In cephalopods, it has been reported that the endocrine optic glands on the optic tract
53 in the brain (central nervous system, CNS) control the gonadal maturation (Boycott and
54 Young, 1956; Wells and Wells, 1959). For detailed information regarding what each
55 region of the CNS comprises their predicted function , including the optic gland in
56 cephalopods, please refer to previous reports (Amano et al., 2008; Cosmo and Cristo,
57 1998; Shigeno and Yamamoto, 2002). The complete sequence of brain GnRH-like was
58 first reported in the mollusc cephalopod octopus, as a candidate endocrine factor
59 regulating reproduction (Iwakoshi et al., 2002). It was also suggested that brain GnRH-
60 like may either regulate gonadal maturation or steroidogenesis via an unknown optic
61 gland hormone such as gonadotropin in vertebrates, or directly in the brain through the

62 gonadal endocrine axis, as reported in octopuses (Cosmo and Cristo, 1998; Iwakoshi-
63 Ukena et al., 2004; Kanda et al., 2006). There are only limited studies on reproduction in
64 other cephalopods such as squids and cuttlefish. GnRH-like has also been identified or
65 reported in the brains of four squid and cuttlefish species using molecular biological
66 methods or immunohistochemistry (IHC) (Amano et al., 2008; Cristo et al., 2009;
67 Onitsuka et al., 2009; Zatylny-Gaudin et al., 2016) (GenBank: KP982885.1). However,
68 to the best of our knowledge, there are no reports to date on the functional reproductive
69 role of GnRH-like in squid or cuttlefish. The expression profile of GnRH-like in the brain
70 with gonadal maturation or differentiation and its relation to the reproductive function of
71 GnRH-like in cephalopods remains unknown.

72 The kisslip cuttlefish, *Sepia lycidas*, belonging to the Sepiidae family, is widely
73 distributed across the Indian Ocean until the West Pacific Ocean (Natsukari and Tashiro,
74 1991). The breeding season for kisslip cuttlefish is from spring to early summer, when
75 they exhibit mating behavior and spawning in coastal areas (Natsukari and Tashiro, 1991).
76 Our previous study exploring gonadal sex differentiation in kisslip cuttlefish showed that
77 ovarian differentiation occurs first marked by the appearance of oocytes before hatching,

78 followed by testicular differentiation as detected by the formation of seminiferous tubules,
79 around 20 days post hatching (DPH) (Murata et al., 2019). However, the physiological
80 mechanism of gonadal sex differentiation in cephalopods, particularly in kisslip cuttlefish,
81 remains elusive. Thus, the aim of this study was to clarify the involvement of GnRH-like
82 in gonadal sex differentiation in the kisslip cuttlefish. In this study, we first identified the
83 kisslip cuttlefish *gnrh-like*/GnRH-like (*kc-gnrh*/kc-GnRH) from the brain, and analyzed
84 its expression profile throughout gonadal sex differentiation by quantitative RT-PCR and
85 IHC. We also performed dual immunostaining using anti-neurofilament, which is a
86 specific marker for mature neurons, to clarify the kc-GnRH immunoreactive (kc-GnRH-
87 ir) cell type in the brain of juvenile kisslip cuttlefish. Simultaneously, the distribution of
88 kc-GnRH immunoreactivity in peripheral tissues apart from the brain was also
89 investigated by IHC to obtain information on the tissue distribution of cephalopod GnRH-
90 like.

91 **2. Material and methods**

92 *2.1 Ethical use of animals*

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

98 *2.2 Animals and sampling procedures*

99 Wild parent kisslip cuttlefish were obtained by line fishing from the shallow coastal
100 area of Nagasaki, or bought from a fish market during their breeding season in 2018 and
101 2019. Fertilized eggs were obtained from parental fishes, and the embryo and juvenile
102 cuttlefish were raised until 30 DPH, under the same conditions and methods as described
103 by Murata et al. (2019). Before hatching, the eggs were dissected to obtain the cuttlefish
104 embryos. After hatching, prior to dissection, juvenile cuttlefish were anesthetized with
105 1.0% ethanol in seawater, following the protocol described by Ikeda et al. (2009). The
106 cuttlefishes were sacrificed by decapitation, and the brain and gonads of 30 cuttlefishes
107 were collected at 14, 21, and 28 days after spawning (DAS), and then at 1, 10, 20, and 30
108 DPH. Whole brains from half of the sacrificed cuttlefishes (15 animals) at each age were

109 fixed in RNA*later*™ Stabilization Solution (Invitrogen, Carlsbad, CA, USA) at 4 °C
110 overnight, and then stored at −30 °C until RNA extraction was performed. The brains
111 from the other half of cuttlefishes (15 animals) and the remaining body of all samples (30
112 animals) including gonads were fixed in Bouin's solution at room temperature (RT) (15–
113 25 °C) overnight, and transferred to 70% ethanol for storage at 4 °C for normal histology
114 or IHC. The brains of six juvenile cuttlefish at 1 DPH were fixed with 4%
115 paraformaldehyde (PFA) at 4 °C overnight, and then transferred to 70% ethanol for
116 storage at 4 °C for immunofluorescence analysis.

117 *2.3 Histological observation of the gonads throughout sex differentiation*

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

122 *2.4 RNA extraction and cloning of kc-gnrh*

123 Total RNA was extracted from the brain samples using the ISOGEN II (Nippon Gene,

124 Tokyo, Japan) following the manufacturer's protocols. RNA concentration was measured
125 using a NanoDrop 2000 Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA)
126 and used further for cloning and quantitative RT-PCR.

127 One microgram total RNA was reverse-transcribed using an oligo (dT) primer and
128 Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) according to
129 the manufacturer's instructions. PCR was performed using degenerate primers (forward:
130 5-taccaytttagcaatggatggcac, reverse: 5-aktttytckatcaaagcytttgtt) designed from a highly
131 conserved region of *gnrh-like* of three cephalopod species (*Octopus vulgaris*:
132 AB037165.1, *Uroteuthis edulis*: AB447557.1, and *Sepiella japonica*: KP982885.1). Tks
133 Gflex DNA polymerase (Takara Bio, Kusatsu, Japan) was used according to the
134 manufacturer's instructions. Amplified products were separated by 2.0% agarose gel
135 electrophoresis and purified from excised gel fragments using a QIAquick Gel Extraction
136 Kit (Qiagen, Hilden, Germany). The isolated sample was ligated with a plasmid vector
137 using a TOPO TA Cloning Kit (Invitrogen) after adding 3′ A-overhangs, and the
138 transformants containing the recombinant plasmid were generated with Competent Quick
139 DH5α cells (Toyobo, Osaka, Japan). Recombinant clones selected by blue-white

140 screening were then used to extract and purify plasmid DNA using the NucleoSpin
141 Plasmid EasyPure kit (MARCHEREY-NAGEL, Düren, Germany), and the purified
142 plasmid was sequenced by FASMAC (Atsugi, Japan). Based on the sequence of the
143 presumptive *kc-gnrh*, Rapid amplification of cDNA ends (RACE) was performed to
144 isolate the 5′ and 3′ ends of the cDNA using SMARTer RACE 5′/3′ kit (Takara
145 Bio) according to the manufacturer's instructions. The mRNA used for RACE was
146 purified from the total RNA using an Oligotex-dT30 <Super> mRNA Purification Kit
147 (Takara Bio). PrimeSTAR Max DNA Polymerase (Takara Bio) was used for RACE PCR.
148 Gene-specific primers (GSPs) in combination with the vector sequence at the 5′ - end
149 (5′-RACE: 5′-gattacgccaagctttggaagtccacttcgtttaccaccag, 3′-RACE: 5′-
150 gattacgccaagcttgcaccctggtggtaaacgaagtggac) and a Universal Primer A Mix, were used
151 for primary PCR. In the 5′ RACE procedure, nested PCR was conducted using nested
152 GSP (5′-gattacgccaagcttccaccagggtgccatccattgctaa) and a Universal Primer Short after
153 the primary PCR.

154 Sequences were analyzed and aligned using the Basic Local Alignment Search Tool
155 (BLAST; https://www.ncbi.nlm.nih.gov) and Multiple Sequence Comparison by Log-

156 Expectation Tool (MUSCLE; https://www.ebi.ac.uk). The functional domain of kc-
157 GnRH was deduced from conserved domains in the other octopus and squid GnRH
158 sequences (Minakata and Tsutsui, 2016).

159 *2.5 Quantification of kc-gnrh*

160 *kc-gnrh* in the brain was quantified using real-time quantitative-PCR. Brain samples
161 (5 to 7 each) from each sexual stage and at each age were reverse-transcribed from 500
162 ng of total RNA in a 10 μL reaction volume, using ReverTra Ace qPCR RT Master Mix
163 with gDNA Remover (Toyobo) according to the manufacturer's instructions. The copy
164 number of *kc-gnrh* was estimated based on the plasmid standard. The primer pair
165 (forward: 5-ggccttttttcacctgtgcta, reverse: 5-tgtctggaagtccacttcgtt, amplicon size: 75 bp)
166 was designed using Primer3Plus (http://www.bioinformatics.nl/cgi-
167 bin/primer3plus/primer3plus.cgi). The dilution templates (corresponding to 50 ng total
168 RNA/well) for each sample were tested in 10 μL of PCR mixture containing 2× KAPA
169 SYBR FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA) and 1 μM
170 of each primer. The PCR protocol was as follows: 1 cycle of initial degeneration at 95 °C
171 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

187 finally washed with phosphate-buffered saline (PBS). The sections were then treated with
188 3% H_2O_2/methanol for 15 min to inactivate endogenous peroxidase activity, incubated
189 with 10% normal goat serum for 15 min to eliminate non-specific binding, and incubated
190 overnight with the primary antibody (diluted 1:4000 in 1% BSA/PBS) in a moist chamber
191 at 4 °C. The primary antibody against kc-GnRH (anti-kc-GnRH) was detected and
192 visualized using the Histofine anti-rabbit IHC kit and diaminobenzidine (Nichirei, Tokyo,
193 Japan), in accordance with the manufacturer's protocol. The histological part of the brain
194 used for IHC was determined as described in Amano et al. (2008). The adjacent sections,
195 considered as control sections, were incubated in the following conditions: original
196 antigen-adsorbed primary antibody (diluted 1:4000 in 1% BSA/PBS, following
197 adsorption with 400-folds of the weight of antigen), solvent alone (1% BSA/PBS), and
198 pre-immunized serum from the rabbit used for antibody generation (diluted 1:4000 in 1%
199 BSA/PBS). No significant background immunoreactive signals were detected in the
200 control group (Supplementary figure 1).

201 Brain samples fixed with 4% PFA for immunofluorescence were sectioned at 5 μm
202 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 (ProLong 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

249 sexual stage-based differences were observed in the distribution of kc-GnRH-ir cell
250 bodies and fibers in the brain throughout gonadal sex differentiation.

251 Dual immunofluorescence showed that kc-GnRH-ir cell bodies were not co-localized
252 with immunoreactivity against anti-NF-M/H in the brain of juvenile kisslip cuttlefish at
253 1 DPH (Fig. 5). The kc-GnRH-ir cells exhibited small shapes. On the other hand, the NF-
254 M/H-immunoreactive cells were larger in shape with clear axons (Fig. 5).

255 *3.5 Distribution of kc-GnRH immunoreactivity in the peripheral tissues of juvenile kisslip*
256 *cuttlefish*

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

260 **4. Discussion**

261 To understand the involvement of the brain GnRH-like in gonadal sex differentiation,
262 we cloned *gnrh-like* from kisslip cuttlefish. Our results clearly indicate that the functional
263 region of GnRH-like is highly conserved among cephalopods, as reported by Minakata

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

275 In the well-studied vertebrate teleost fish, gonadal sex differentiation is mainly
276 triggered by a sex steroid hormone which is secreted from the gonads, with limited
277 involvement of the pituitary gonadotropins or brain GnRH (Yan et al., 2012). On the other
278 hand, it has been reported that GnRH-like directly stimulates gonadal gametogenesis in
279 the molluscs scallop *Patinopecten yessoensis,* and in the abalone *Haliotis asinina,*

suggesting its direct involvement in gonadal sex differentiation (Nagasawa 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 (Ijiri 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

296 present study collectively conclude that the ovarian differentiation period, shows
297 temporary activation of brain kc-GnRH in both sexes, and that this occurs before hatching,
298 which is the starting period for feeding or moving during early ontogenesis (Murata et al.,
299 2019). From these facts, we assume that the activated kc-GnRH during the ovarian
300 differentiation period may have some functional role, not on gonadal differentiation, but
301 on the initiation of feeding or moving. Further analysis of the GnRH-like receptor is
302 required to clarify the involvement of GnRH-like as a direct regulating factor of early
303 ontogenesis, including gonadal sex differentiation in kisslip cuttlefish.

304 It has already been proved by the surgical removal experiment that gonadal
305 maturation in the cephalopod octopus is neurally controlled by the optic gland, which is
306 considered to be a neuroendocrine organ in the brain, as summarized by Cosmo and Cristo
307 (1998). Subsequently, GnRH-like immunoreactivity was demonstrated in the regulating
308 area of the optic gland, suggesting the critical role of GnRH-like as an upstream regulator
309 of reproduction in octopus (Cosmo and Cristo, 1998). In the adult spear squid, GnRH-
310 like immunoreactivity was also detected around the optic gland area, as well as in the pvL
311 and vmL, giving rise to the assumption that GnRH-like may regulate reproduction

312 through the optic gland (Amano et al., 2008). Thus, in the case of adult cephalopods,
313 GnRH-like usually shows active immunoreactivity around the optic gland suggesting a
314 role in regulating reproduction. However, the present study is the first to demonstrate the
315 location of kc-GnRH-ir cell bodies only in the pvL and vmL in the brain of kisslip
316 cuttlefish at an early developmental stage, and not in the optic gland area. A
317 morphological study of the pygmy cuttlefish, *Idiosepius paradoxus*, summarized that the
318 major function of pvL and vmL in the brain is presumably not in reproduction, but muscle
319 control, ink emission, or jet propulsion (Shigeno and Yamamoto, 2002). In addition, our
320 present study demonstrates for the first time that kc-GnRH-ir cell bodies in the brain are
321 not colocalized with neurofilaments, which is the specific structure for mature neurons,
322 including neuroendocrine neurons (Lee et al., 1988, 1987). This result indicates that kc-
323 GnRH is distributed in glial cells or immature neurons and mainly plays a supporting role
324 for functional neurons during early ontogenesis (Freeman, 2015). In the case of adult
325 octopus or squid, GnRH-like immunoreactivity was observed in neurons with active
326 fibers in the brain, regulating reproduction as a neuroendocrine factor (Amano et al.,
327 2008; Cosmo and Cristo, 1998; Cristo et al., 2009). These observations indicate that the

328 function of the endocrine axis consisting of the optic gland and gonad, might control
329 reproduction in cephalopods, might be immature in the early developmental stages of the
330 kisslip cuttlefish. Consequently, it is unlikely that brain kc-GnRH is involved as an
331 upstream factor in gonadal sex differentiation. There is limited definitive knowledge
332 regarding the function of each part of the brain as well as the endocrine axis from the
333 optic gland to the gonad, regulating reproduction in squid or cuttlefish; therefore, further
334 studies are needed to conclusively define the involvement of GnRH-like in sex
335 differentiation.

336 Molecular evolutionary and phylogenetic studies on vertebrate and invertebrate
337 GnRH recently revealed that GnRH and GnRH-like belong to a larger peptide family
338 consisting of GnRH, adipokinetic hormone, corazonin, and adipokinetic
339 hormone/corazonin-related peptides, and that these peptides might share a common
340 ancestor (Lindemans et al., 2010; Tsai, 2018; Tsai and Zhang, 2008; Zandawala et al.,
341 2018). Additionally, several reports have indicated a functional diversity of GnRH and
342 GnRH-like with the evolution of not only reproductive roles but also cardiac regulatory
343 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.

5. Declaration of Competing Interest

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

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Figure captions

Figure 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 μm.

Figure 2. Alignment of precursors of cephalopod GnRH-like peptides. Shaded regions are the conserved functional regions of GnRH.

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

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

390 palliovisceral lobe; vmL, ventral magnocellular lobe. Scale bars = 20 μm.

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

396 **Supplementary figure 1.** Adjacent cross sections of juvenile kisslip cuttlefish (20 DPH)
397 palliovisceral lobe of the brain immunostained with anti-kc-GnRH (a), anti-kc-GnRH-
398 adsorbed primary antibody (b), 1% BSA/PBS (c), and pre-immunized serum (d).
399 Arrowheads in (a) indicate GnRH immunoreactive cell bodies. DPH, days post hatching.
400 Scale bars = 20 μm.

401 **Supplementary figure 2.** Adjacent cross sections of juvenile kisslip cuttlefish (10 DPH)
402 heart immunostained with anti-kc-GnRH (a), and anti-kc-GnRH-adsorbed primary
403 antibody (b). Arrowheads in (a) indicate GnRH immunoreactive cell bodies. Au, auricle;
404 Liv, liver; DPH, days post hatching. Scale bars = 20 μm.

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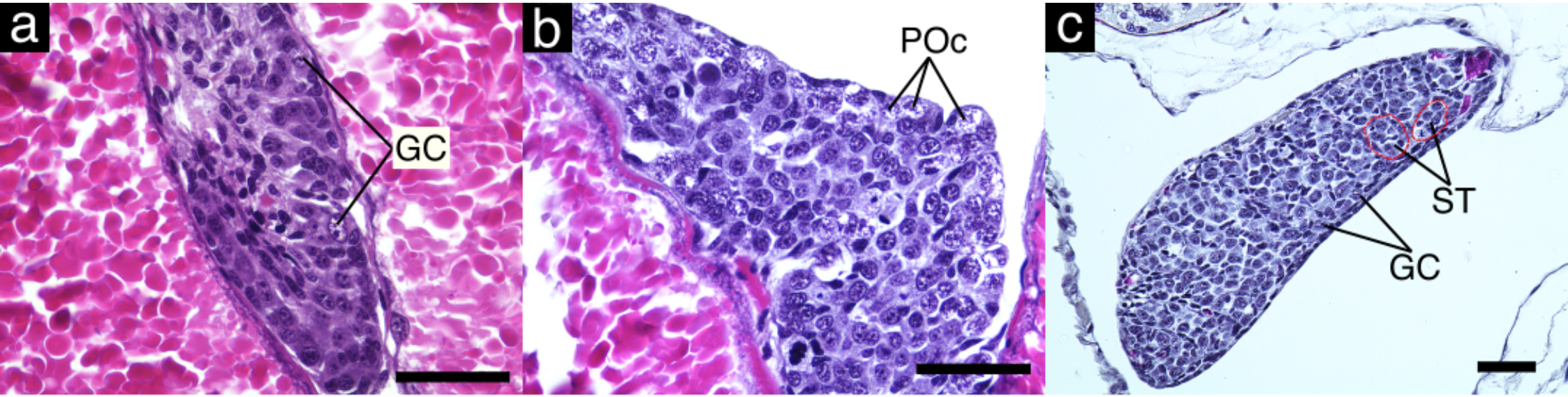

Figure 1


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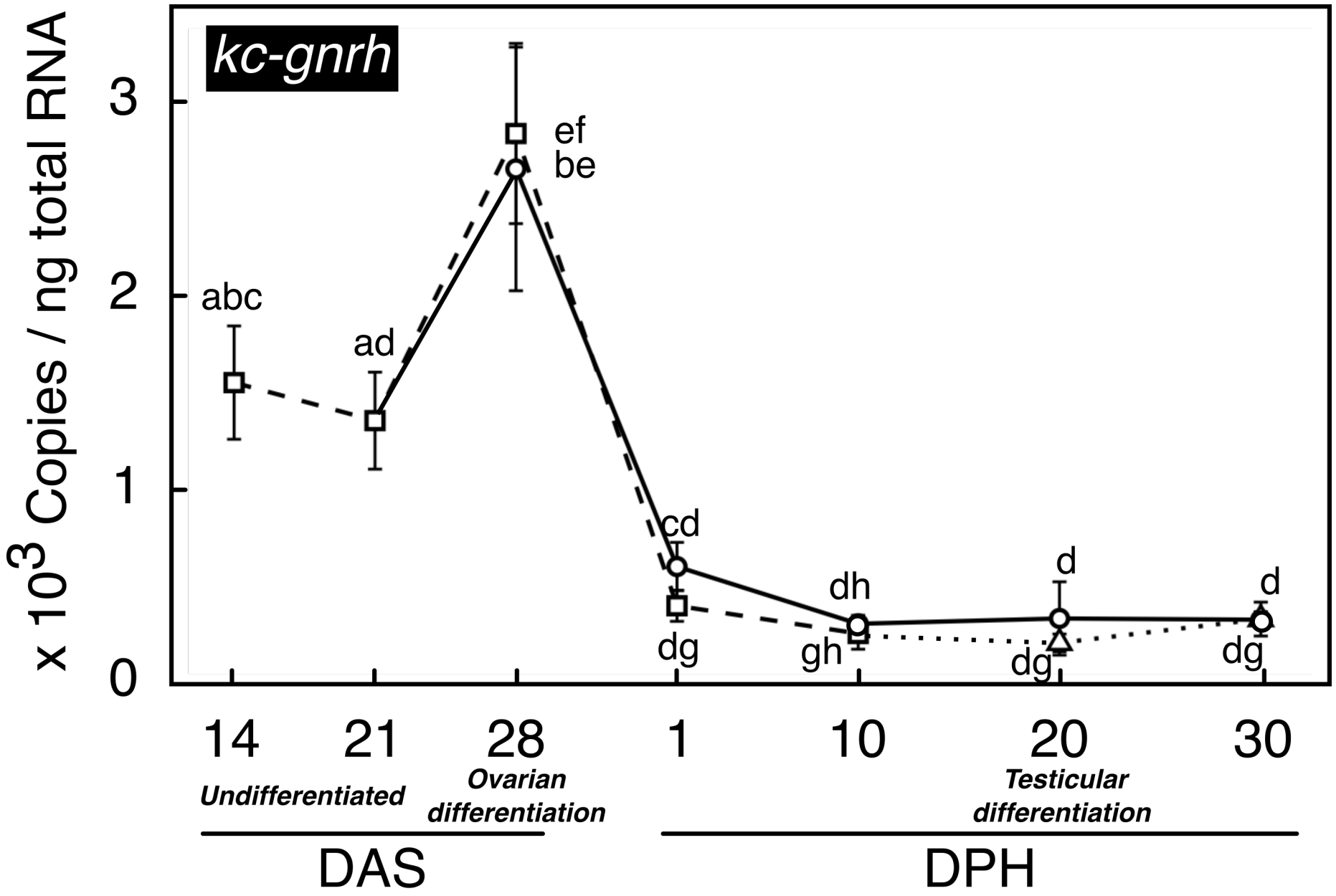
Figure 2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Ovarian differentiation
Testicular differentiation
DAS
DPH

Figure 3

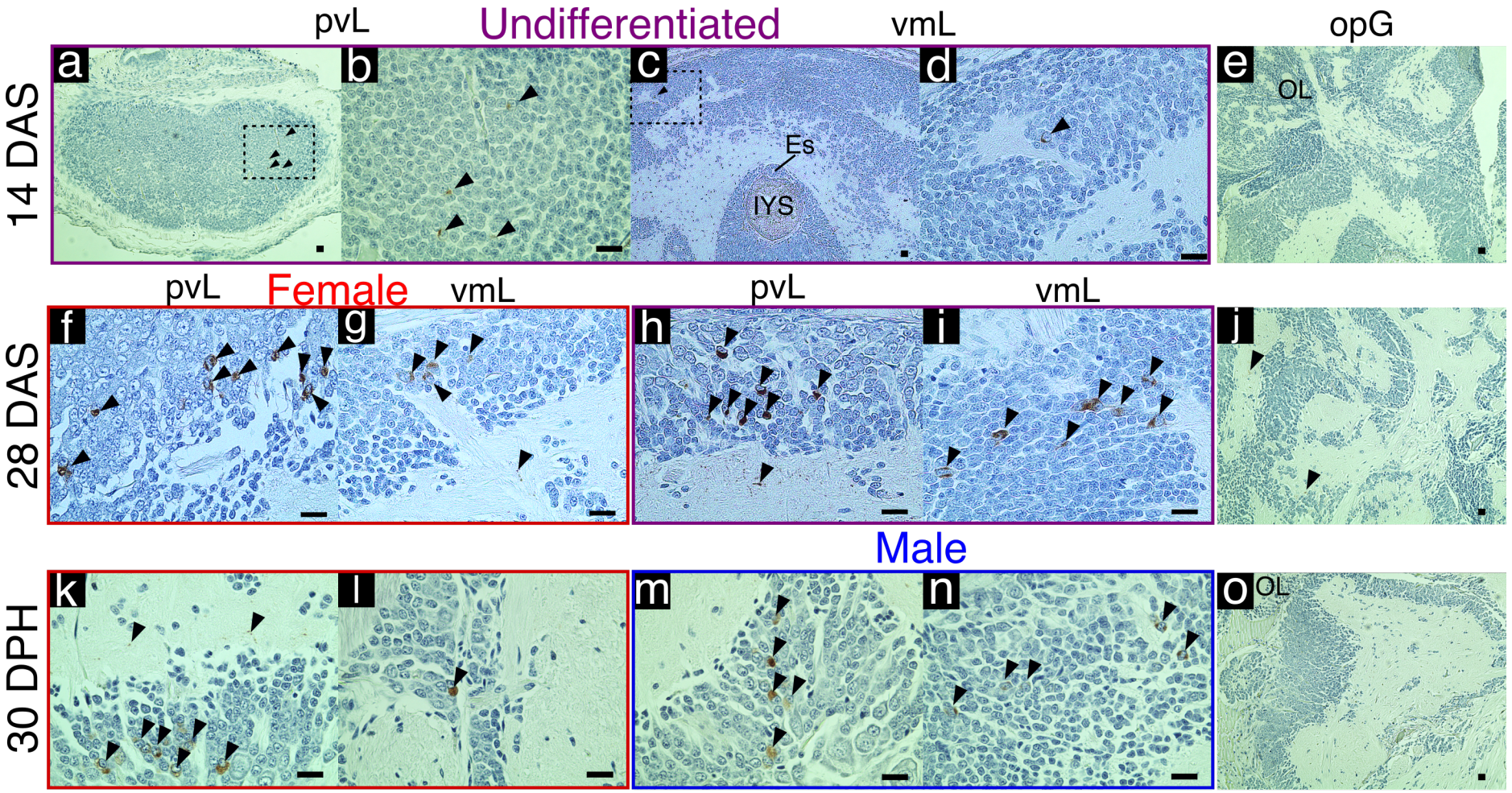
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Figure 4

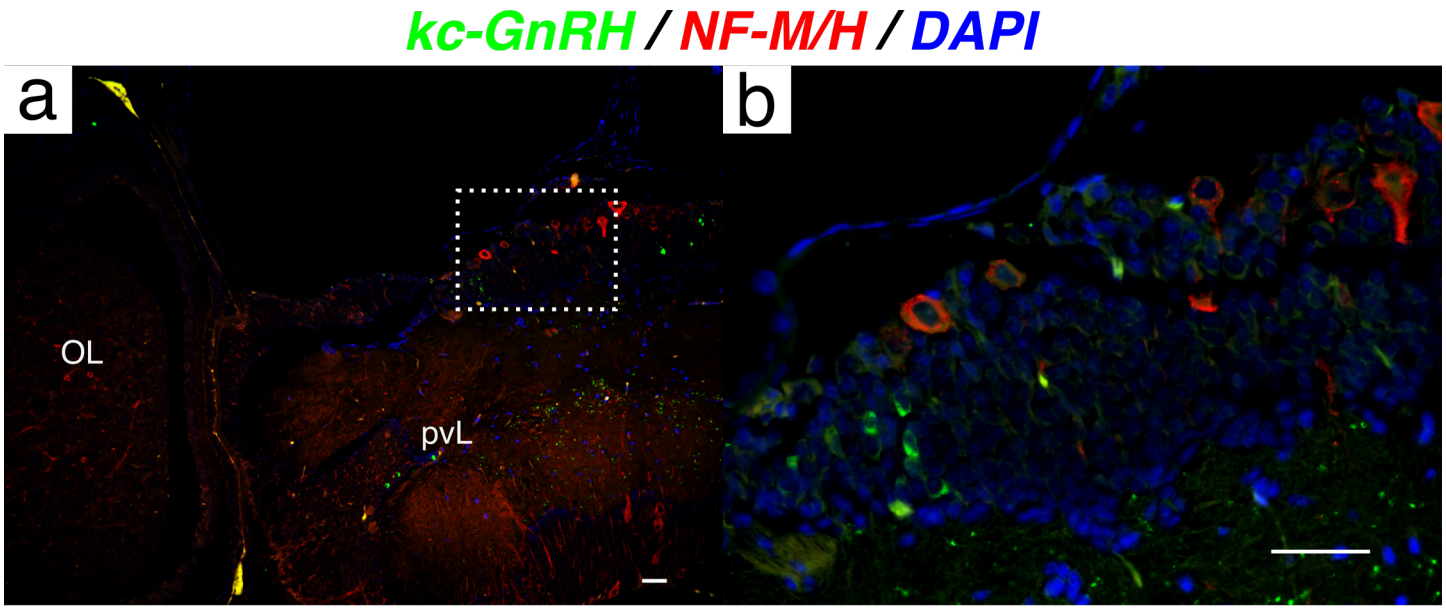
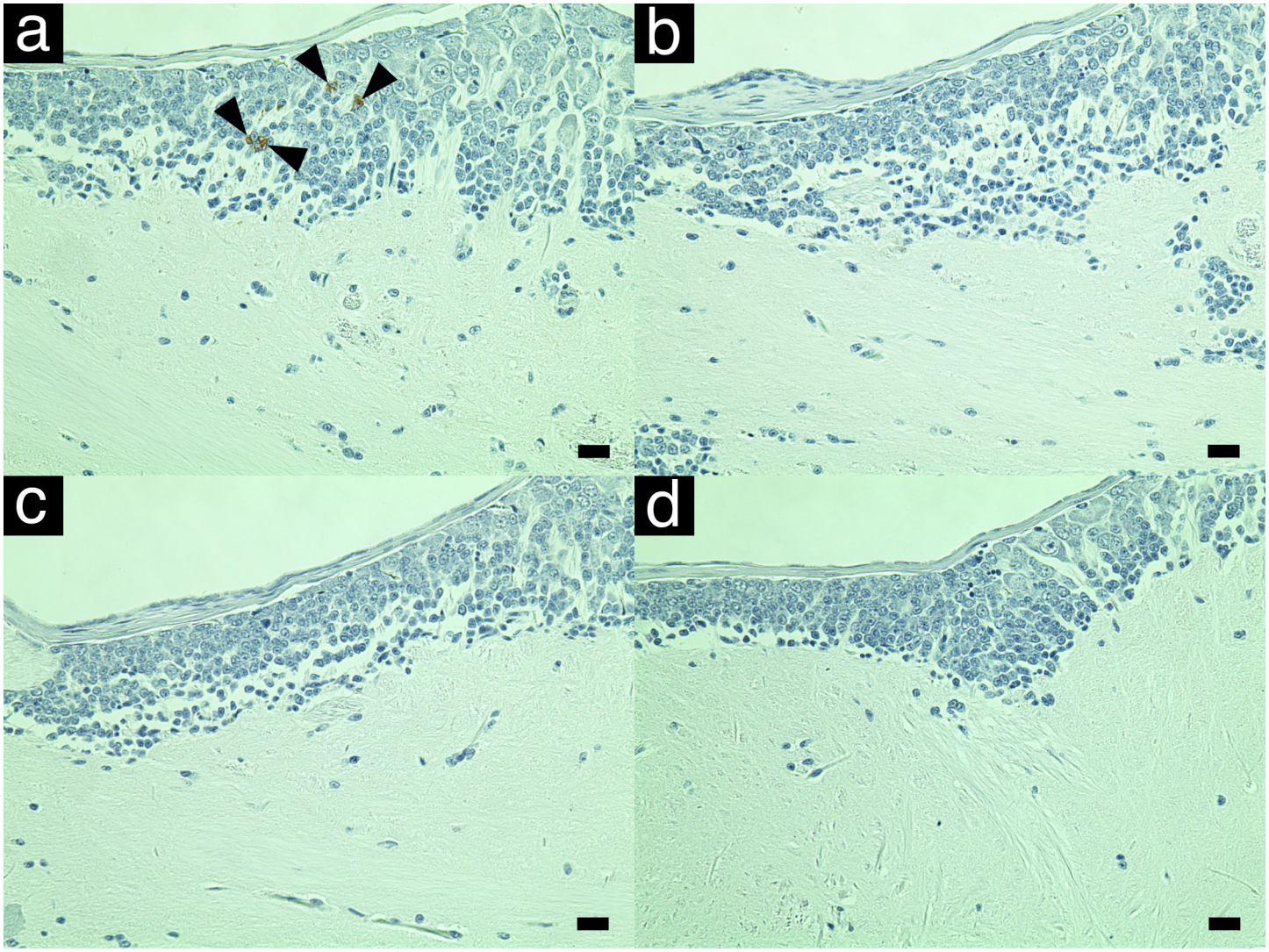
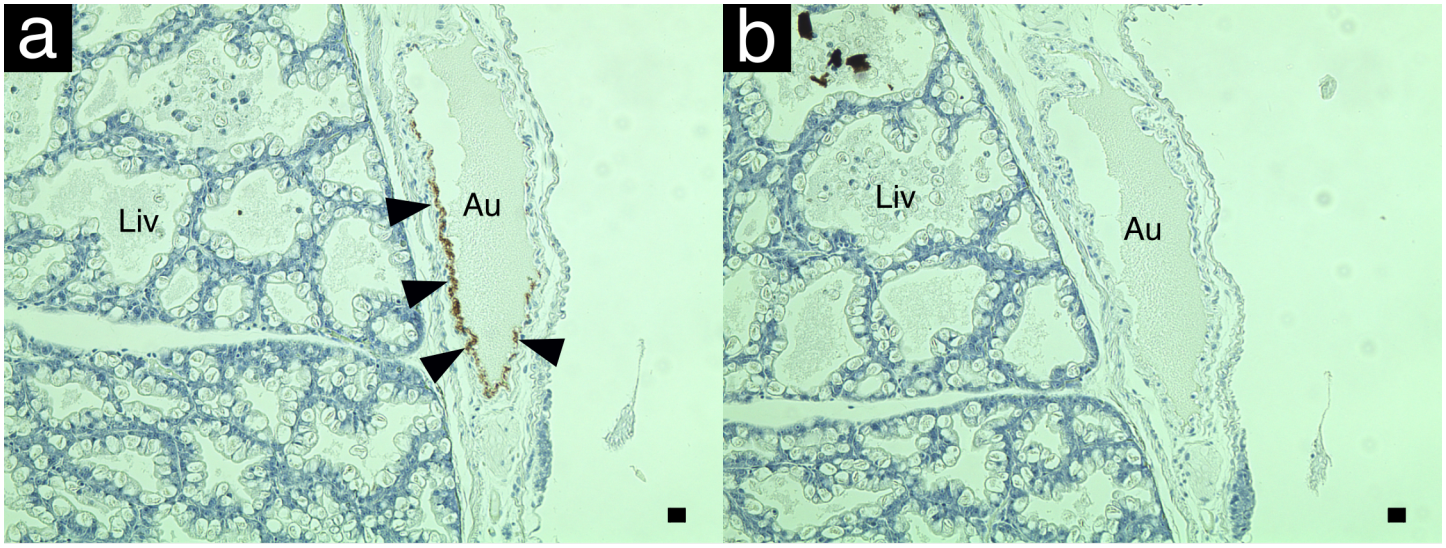


Figure 5



Supplementary figure 1



Supplementary figure 2