Localization of three forms of gonadotropin-releasing hormone in the brain and pituitary of the self-fertilizing fish, *Kryptolebias marmoratus*

Masafumi Amano[1*], Noriko Amiya[1], Kataaki Okubo[2], Junpei Yamashita[2], Ayae Kuriu[1], Ayano Yasuta[1], Naoyuki Yamamoto[3], Yoshitaka Sakakura[4]

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

[2] Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo, Tokyo 113-8657, Japan

[3] Laboratory of Fish Biology, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Aichi 464-8601, Japan

[4] Graduate School of Fisheries and Environmental Sciences, Nagasaki University, Nagasaki 852-8521, Japan

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[*]Corresponding author: Tel & Fax: +81-42-778-8884

E-mail: amanoma@kitasato-u.ac.jp

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Abstract

The localization of gonadotropin-releasing hormone (GnRH) in the brain and pituitary of the self-fertilizing mangrove killifish *Kryptolebias marmoratus* was examined by immunohistochemistry and *in situ* hybridization to understand its neuroendocrine system. The genome assembly of *K. marmoratus* did not have any sequence encoding GnRH1, but sequences encoding GnRH2 (chicken GnRH-II) and GnRH3 (salmon GnRH) were found. Therefore, GnRH1 was identified by *in silico* cloning. The deduced amino acid sequence of the *K. marmoratus* GnRH1 (mature peptide) was identical to that of the medaka GnRH. GnRH1 neurons were detected in the ventral part of the preoptic nucleus (PO) by immunohistochemistry and *in situ* hybridization and GnRH1-immunoreactive (ir) fibers were observed throughout the brain. GnRH1-ir fibers were in close contact with luteinizing hormone (LH)-ir cells in the pituitary using double immunohistochemistry. GnRH2 neurons were detected in the midbrain tegmentum by immunohistochemistry and *in situ* hybridization. Although GnRH2-ir fibers were observed throughout the brain, they were not detected in the pituitary. GnRH3 neurons were detected in the lateral part of the ventral telencephalic area by both methods. GnRH3-ir fibers were observed throughout the brain, and a few GnRH3-ir fibers were in close contact with LH-ir cells in the pituitary. These results indicate that GnRH1 and possibly GnRH3 are responsible for gonadal maturation through LH secretion and that all three forms of GnRH function as neurotransmitters or neuromodulators in the brain of *K. marmoratus*.

Introduction

The mangrove killifish *Kryptolebias marmoratus* (Cyprinodontiformes) formerly named *Rivulus marmoratus* is the sole known vertebrate species that utilizes self-fertilization for reproduction (Harrington, 1961). *K. marmoratus* is sexually dimorphic; sexually mature fish are either hermaphrodite or male (no mature females have been reported). Hermaphrodite *K. marmoratus* have functional ovary and testis (ovotestis), whereas male fish have functional testis only (Harrington, 1967; Soto et al., 1992). There are two types of males: primary males, which often appear from exposure to low water temperatures during embryonic development, and secondary males, which arise from hermaphrodite fish by ovary regression (Harrington, 1967, 1971). It has been suggested that immature individuals (less than 60 days after hatching) are females, and the percentage of hermaphrodite fish increases with age (Cole and Noakes, 1997; Sakakura and Noakes, 2000). Thus, the reproductive biology of *K. marmoratus* is unique (Minamimoto et al., 2006).

Plasma steroid hormone levels and steroidogenesis in the gonad have been examined in *K. marmoratus* reproduction by enzyme linked immunosorbent assay (Minamimoto et al., 2006). Estradiol-17β and 11-ketotestosterone were detected in the plasma of hermaphrodite and primary male fish. Early and late ovarian follicles of hermaphrodite fish and the testis of primary males synchronously secreted estradiol-17β, 11-ketotestosterone, and 17α,20β-dihydroxy-4-pregnen-3-one after incubation in medium with 17α-hydroxyprogesterone or testosterone. Thus, both hermaphrodite fish and primary male fish secrete estrogen, androgen, and progestin synchronously.

The secretion of steroid hormones from the gonads is controlled by pituitary gonadotropins (GTHs), and GTH secretion is regulated by gonadotropin-releasing hormone (GnRH) (see Okubo and Nagahama, 2008). Because *K. marmoratus* hermaphrodites maintain both a functional ovary and testis, and androgenic and estrogenic steroid hormones are secreted synchronously (Minamimoto et al., 2006), we hypothesized that there may be a unique hormonal regulation system

in the pituitary gland of *K. marmoratus* that controls the secretion of sex steroid hormones. The β-subunit genes of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were cloned (Rhee et al., 2009), and the GnRH receptor (GnRHR) gene was identified and has typical vertebrate GnRHR domains and motifs (Rhee et al., 2008).

Several forms of GnRH have been identified based on amino acid sequences or complementary DNA in vertebrates, and two or three forms of GnRH exist even within the same species in teleosts (see Okubo and Nagahama, 2008). GnRH2 (chicken GnRH-II) is present throughout vertebrates, from cartilaginous fish to some species of mammals.

K. marmoratus belongs to the Cyprinodontiformes. A taxonomically close species, mummichog *Fundulus heteroclitus* (Cyprinodontiformes), has three forms of GnRH including GnRH1 (medaka GnRH), GnRH2, and GnRH3 (salmon GnRH) (Ohkubo et al., 2010). Thus, *K. marmoratus* may also have these three forms. Recently, mRNA sequences encoding GnRH2 and GnRH3 in *K. marmoratus* have been updated in a publicly available database (NCBI database). Thus, we aimed to identify GnRH1 in the *K. marmoratus* genome by *in silico* cloning. Here, we examined the localization of GnRH1, GnRH2 and GnRH3 in the brain and pituitary of *K. marmoratus* by immunohistochemistry, and neurons expressing GnRH mRNA were detected by *in situ* hybridization. Finally, the anatomical interactions between GnRH neuronal fibers and GTH cells in the pituitary were analyzed by double immunohistochemistry.

Materials and methods

In silico cloning of K. marmoratus GnRHs

The *K. marmoratus* genome assembly (ASM164957v1 reference Annotation Release 100) was TBLASTN-searched using precursor amino acid sequences of known GnRHs in other teleost species including mummichog GnRH1 (GenBank accession number: NP_001296933) and medaka GnRH1 (GenBank accession number: NP_001098169).

In addition, the unassembled whole genome shotgun sequence data (100 bp paired-end reads) of *K. marmoratus* (SRA accession number: SRX1074415) were searched by BLASTN using the nucleotide sequences of the mummichog (GenBank accession number: NM_001310004) and medaka (GenBank accession number: NM_001104699) GnRH1 cDNAs. This BLASTN search was repeated dozens of times using newly identified sequences as queries to obtain the full length sequence of the open reading frame. Subsequently, the unassembled RNA-seq datasets (reads of 50 bp length) of *K. marmoratus* (SRA accession numbers: SRX1011289, SRX1012828, SRX1012832, SRX1012838, SRX1012842) were searched by BLASTN using the genome sequence obtained above as queries to determine exon and intron boundaries.

The deduced amino acid sequence of each *K. marmoratus* GnRH precursor identified was aligned with the orthologs in mummichog and medaka *Oryzias latipes*. The sequences and GenBank accession numbers used in the alignment were as follows: mummichog GnRH1, NP_001296933; medaka GnRH1, NP_001098169; *K. marmoratus* GnRH2, XP_017278278; mummichog GnRH2, BAF96396; medaka GnRH2, NP_001098141; *K. marmoratus* GnRH3, XP_017287543; mummichog GnRH3, BAF95685; medaka GnRH3, NP_001098142.

The deduced amino acid sequences of GnRH precursors in *K. marmoratus* were aligned with those in other teleost species. The resulting alignment was used to construct a bootstrapped (1000 replicates) neighbor-joining phylogenetic tree (http://clustalw.ddbj.nig.ac.jp/index.php?lang=en).

The sequences of GnRH precursors in sea hare *Aplysia californica* and octopus *Octopus vulgaris* were used as outgroups. The sequences and GenBank accession numbers used in the phylogenetic analysis were as follows: flounder *Verasper moseri* GnRH1, BAB83984; seabream *Sparus aurata* GnRH1, AAA75469; cichlid *Astatotilapia burtoni* GnRH1, NP_001273225; mummichog GnRH1, NP_001296933; medaka Gnrh1, NP_001098169; eel *Anguilla japonica* Gnrh1, BAA82608; flounder GnRH2, BAB83983; seabream GnRH2, AAA75447; cichlid GnRH2, AAA74993; *K. marmoratus* GnRH2, XP_017278278; mummichog GnRH2, BAF96396; medaka GnRH2, NP_001098141; zebrafish *Danio rerio* GnRH2, NP_852104; eel GnRH2, BAA82609; flounder GnRH3, BAB83982; seabream GnRH3, AAA98845; cichlid GnRH3, NP_001273267; *K. marmoratus* GnRH3, XP_017287543; mummichog GnRH3, BAF95685; medaka GnRH3, NP_001098142; zebrafish GnRH3, NP_878307; sea hare GnRH, NP_001191482; octopus GnRH, BAB86782.

Fish

Hermaphrodites from a single clonal lineage of *K. marmoratus* (body weight, 0.024–0.084g; age, 114–1588 days after hatching) were used. This lineage originated from one fish collected in Dangriga, Belize, and has been maintained for more than 10 generations at the Aquaculture Biology Laboratory at Nagasaki University (Kanamori et al., 2016). Under laboratory conditions, this species matures 90 days after hatching (Sakakura and Noakes, 2000), and the lifespan is 3-5 years with some animals living up to 8 years (Taylor, 2012). Spawning occurs throughout the year, and there is no distinct spawning season or diel rhythm (Grageda et al., 2005). The fish were held individually in translucent plastic containers with 60 mL of brackish water (17 ppt) and were reared under a controlled photoperiod (LD 14:10) and temperature (25 °C). Fish were fed *Artemia franciscana* nauplii until satiation three times a week. The spawning of individual viable eggs were recorded separately, and we confirmed that all specimens used were mature adults. Fish

maintenance and sacrifice were performed following the guidelines of the animal care committee of Kitasato University.

GnRH immunohistochemistry

Fish were anesthetized by immersion in 0.05 % 2-phenoxyethanol. Head regions were immediately fixed with Bouin's fluid for 24 hours at 4 ºC and subsequently rinsed in cold 70% ethanol, dehydrated through a graded series of ethanol concentrations and embedded in paraplast. Serial sagittal or frontal sections were cut at 5 or 8 μm, and mounted on MAS-GP coated slides (Matsunami, Osaka, Japan).

Immunohistochemistry was conducted according to Amano et al. (2016). Antibody information including antibody types (polyclonal or monoclonal), species used for antibody generation, antigen sequence, carrier molecule, cross-reactivity to GnRH forms and source of antibody is summarized in Table 1. Rabbit polyclonal antibodies raised against GnRH1 (lot no. 2), GnRH2 (aCII6), and GnRH3 (Lot no. 2) were used. The antiserum against GnRH1 cross-reacts with GnRH2 and GnRH3 by less than 0.8% (Karigo et al., 2012). The antiserum against GnRH2 cross-reacts 0.05% and 0.01% with GnRH3 and lamprey GnRH−I, respectively. The antiserum against GnRH3 cross-reacts 1.58% and 0.08% with GnRH2 and lamprey GnRH−I, respectively. Both GnRH2 and GnRH3 antisera do not cross-react with mammalian GnRH and cGnRH-I (Senthilkumaran et al., 1999). Antibodies to GnRH1, GnRH2, and GnRH3 were diluted 2,000-fold in 0.1 M phosphate buffer (pH 7.4) containing 0.75% NaCl and 0.3% Triton X-100 (PBST). For immunohistochemical reactions, a Histofine immunostaining kit (Nichirei, Tokyo, Japan) was used. Because GnRH1 immunoreactivity was not observed by this immunohistochemical method, we used the Liberate Antibody Binding Solution (Polysciences Inc, Warrington, PA, USA) to expose the antigenic site for GnRH1 immunohistochemistry.

To test immunoreaction specificity, each antiserum was pre-absorbed overnight at 4 °C with an

167 excess amount of corresponding synthetic GnRH peptide, e.g., 2.5 μg of GnRH1 in 1 mL of diluted
168 GnRH1 antiserum. Because antibodies raised using keyhole limpet hemocyanin (KLH) as a
169 conjugate tend to cross-react with unrelated antigens in fixed samples, the anti-GnRH1 antiserum
170 was also pre-absorbed overnight at 4 °C with an excess amount of KLH (200 μg of KLH in 1 mL of
171 diluted GnRH1 antiserum).
172 For the histological identification of nuclear boundaries, adjacent sections were stained with
173 cresyl violet. We followed the terminology for brain nuclei of Wullimann et al. (1996).
174
175 *In situ* hybridization for GnRH
176
177 Fish were anesthetized by immersion in 0.05 % 2-phenoxyethanol. Head regions were immediately
178 fixed with 4% paraformaldehyde (PFA) for 24 hours at 4 °C and subsequently rinsed in cold 70%
179 ethanol, dehydrated through a graded series of ethanol concentrations and embedded in paraplast.
180 Serial sagittal or frontal sections were cut at 8 or 10 μm, and mounted on Adhesive Glass Slide
181 CRE-01 (Matsunami).
182 *In situ* hybridization was conducted according to Hiraki et al. (2012). Sections were digested
183 with proteinase K (Wako Pure Chemical Industries, Osaka, Japan) for 15 min at 37 °C, post-fixed
184 with 4% PFA for 10 min, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine for
185 15 min. Hybridization was conducted overnight at 55 °C with the DIG-labeled RNA probes (347-bp,
186 370-bp, and 403-bp probes for *gnrh1*, *gnrh2*, and *gnrh3*, respectively) in hybridization buffer: 50%
187 formamide, 5× saline-sodium citrate (SSC), 5× Denhardt's solution, 2 mg/mL yeast RNA and 30
188 μg/mL calf thymus DNA. The sections were washed in 5× SSC, 50% formamide for 20 min at 55
189 °C and in 2× SSC twice for 20 min at 55 °C. The hybridized probes were visualized using alkaline
190 phosphatase-conjugated anti-DIG Fab fragment (Roche, Penzberg, Germany) in a dilution of
191 1:2,000 and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Roche,
192 Penzberg, Germany) following the manufacturer's instructions. The color was allowed to develop

overnight in the dark. The sections were post-fixed with 4% PFA for 15 min, rinsed in distilled water, and mounted with Aqua-Poly/Mount (Polysciences, Inc, Warrington, PA, USA).

Double immunohistochemistry of GnRH and GTH

Because GnRH1-immunoreactive (ir) fibers and GnRH3-ir fibers were detected in the pituitary, anatomical interactions between GnRH1-ir fibers and GTH-ir cells and between GnRH3-ir fibers and GTH-ir cells in the pituitary were investigated by double immunohistochemistry. Universal antisera raised against synthetic fragment peptides corresponding to conservative regions of mummichog FSHβ 50-60 (Lot. 003) and mummichog LHβ 91-106 (Lot. 299) (Shimizu et al., 2003) were used. Both FSH-ir cells and LH-ir cells were successfully identified by these antibodies in the fish of almost every order of the superorder Acanthopterygii and several species of the superorders Paracanthopterygii and Polymixiomorpha (Shimizu et al., 2003). The sequence homology of the corresponding *K. marmoratus* FSHβ to mummichog FSHβ (50-60) is 91%, and that of the corresponding *K. marmoratus* LHβ to mummichog LHβ (91-106) is 75% (Rhee et al., 2009). To test the specificity of the immunoreactions, each antiserum was pre-absorbed overnight at 4 °C with an excess amount of corresponding GTH peptide, e.g., 2.5 μg of mummichog LHβ 91-106 in 1mL of diluted antiserum (Lot. 299).

For double immunohistochemistry, we followed the immunohistochemical procedure previously described in Amano et al. (2016) with a slight modification. After reacting with anti-GnRH antibodies (anti-GnRH1, lot no. 2 and anti-GnRH3, Lot. No. 2) and developing with 3,3'-diaminobenzidine, sections were kept in 0.1 M glycine-HCl buffer (pH 2.0) and then in 8 M urea solution at 40 °C for 60 min to prevent possible interactions between the first and second staining system. Subsequently, the sections were washed three times with 0.1 M PBST and incubated overnight at 4 °C with anti-GTH antibody diluted 2,000-fold in 0.1 M PBST containing 0.02% BSA. Biotin-labeled anti-rabbit IgG solution was added to the sections after rinsing three

219 times with 0.1 M PBST. This was followed by re-rinsing the sections three times with 0.1M PBST,
220 and alkaline phosphatase-labeled streptavidin solution (PerkinElmer Life Sciences Japan, Tokyo,
221 Japan) diluted 100-fold in 0.1 M PBST was added. After three washes in 0.1 M PBST, nitro blue
222 tetrazolium chloride, and 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (NBT/BCIP stock
223 solution; Roche) diluted 50-fold in a solution of 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, and 0.05 M
224 $MgCl_2$ was added to sections to facilitate the visualization of alkaline phosphatase. Finally, the
225 sections were rinsed in distilled water and mounted with Aqua-Poly/Mount.
226

Results

In silico cloning of K. marmoratus GnRHs

TBLASTN searches of the *K. marmoratus* genome assembly identified the GnRH2 gene (gene ID: 108239835; transcript ID, XM_017422789; protein ID: XP_017278278) in the scaffold NW_016094307 and the GnRH3 gene (gene ID: 108245287; transcript ID, XM_017432054; protein ID: XP_017287543) in the scaffold NW_016094243, but not the GnRH1 gene. Additional searches in the unassembled whole genome shotgun and RNA-seq sequence data of *K. marmoratus* allowed identification of the GnRH1 gene and its mRNA sequence (Fig. 1A).

The deduced amino acid sequences of the *K. marmoratus* GnRH1, GnRH2, and GnRH3 precursors aligned with their orthologs in mummichog and medaka are shown in Figure 1B. As expected, each *K. marmoratus* GnRH precursor had high homology to the mummichog and medaka GnRH orthologs.

Phylogenetic analysis confirmed that *gnrh1*, *gnrh2*, and *gnrh3* in *K. marmoratus* were orthologous to the corresponding genes in other teleost species (Fig. 2).

GnRH immunohistochemistry

The distribution of GnRH1-ir cell bodies and fibers is summarized in Figures 3 and 4. GnRH1-ir cell bodies were detected in the ventral part of the preoptic nucleus (PO) (Figs. 3A, 4C, 5A, B, E, F). In some cases, GnRH1-ir cell bodies were also detected in the lateral part of the ventral telencephalic area (Vl) (Fig. 5G). GnRH1-ir fibers were observed throughout the brain; they were abundant in the telencephalon, the hypothalamus, and the medulla oblongata (Figs. 3, 4). No GnRH1-ir cell bodies and fibers were observed when the anti-GnRH1 antibody was pre-absorbed overnight at 4 °C with an excess amount of GnRH1 (Fig. 5C). However, GnRH1-ir cell bodies and

253 fibers did not disappear when the anti-GnRH1 antibody was pre-absorbed overnight at 4 °C with an
254 excess amount of KLH (Fig. 5D). In the pituitary, GnRH1-ir fibers were detected in the
255 neurohypophysis (NH) and invaded the proximal pars distalis (PPD) of the adenohypophysis (Fig.
256 5H, I, L, M). Non-fibrous structure in the rostral pars distalis (RPD), PPD, and pars intermedia (PI)
257 was a result of non-specific staining because these structures did not disappear when using a
258 pre-absorption antibody with synthetic GnRH1 (Fig. 5J, K).
259 The distribution of GnRH2-ir cell bodies and fibers is summarized in Figures 6 and 7.
260 GnRH2-ir cell bodies were located in the midbrain tegmentum (MT) close to the nucleus of the
261 medial longitudinal fascicle (NFLM) (Figs. 6A, 7E, 8A, B, C, D). In some individuals, they were
262 also detected in the dorsal part of the PO (Figs. 6B, 7C, D, 8E, F). Localization of GnRH1-ir cell
263 bodies and that of GnRH2-ir cell bodies in the PO was different; GnRH1-ir cell bodies and
264 GnRH2-ir cell bodies were located in the ventral part of the PO and the dorsal part of the PO,
265 respectively. GnRH2-ir fibers were observed throughout the brain; they were abundant in the
266 telencephalon, the midbrain and the medulla oblongata (Figs. 6, 7). No GnRH2-ir cell bodies and
267 fibers were observed when the anti-GnRH2 antibody was pre-absorbed overnight at 4 °C with an
268 excess amount of GnRH2 (Fig. 8G, H). Incidentally, dense staining was detected in the PI of the
269 pituitary (Fig. 8A), and this staining disappeared by the pre-absorption antibody (data not shown).
270 Because the staining was non-fibrous and GnRH2-ir fibers were sparse in the hypothalamus, this
271 staining may be the result of a cross-reaction of the antibody with an unknown substance.
272 The distribution of GnRH3-ir cell bodies and fibers is summarized in Figures 9 and 10.
273 GnRH3-ir cell bodies were detected in the Vl (Figs. 9A, 10B, 11A, B). In some cases, they were
274 detected also in the lateral tuberal nucleus (NLT) (Figs. 9B, 10E, 11C, D). GnRH1-ir cell bodies and
275 GnRH3-ir cell bodies in the Vl were clearly distinguishable; GnRH1-ir cell bodies were round and
276 larger than GnRH3-ir cell bodies. GnRH3-ir fibers were observed throughout the brain; they were
277 abundant in the telencephalon, the hypothalamus, and the medulla oblongata (Figs. 9, 10).
278 Projections of GnRH3-ir fibers to the pituitary were not evident; only a few GnRH3-ir fibers were

detected in the pituitary (Figs. 10F, 11E, F). No GnRH3-ir cell bodies and fibers were observed when the anti-GnRH3 antibody was pre-absorbed overnight at 4°C with an excess amount of GnRH3 (Fig. 11G, H).

In situ hybridization for GnRH

gnrh1-expressing neurons were detected in the ventral part of the PO (Fig. 12A, B). No *gnrh1*-expressing cells were observed in the pituitary (Fig. 12A). *gnrh2*-expressing neurons were detected only in the MT (Fig. 12C, D), and no *gnrh2*-expressing cells were detected in the pituitary (Fig. 12C). *gnrh3*-expressing neurons were detected in the Vl (Fig. 12E, F) and the ventromedial thalamic nucleus (VM) (Fig. 12E, G). No *gnrh3*-expressing cells were detected in the pituitary (data not shown).

Double immunohistochemistry of GnRH and GTH

No FSH-ir cells were detected in both control and pre-absorption sections (data not shown). LH-ir cells were detected in the PPD and PI (Fig. 13A). No LH-ir cells were detected when the anti-LHβ antibody was pre-absorbed overnight at 4 °C with an excess amount of synthetic LHβ (Fig. 13B). Using double immunohistochemistry for GnRH1 and LH, GnRH1-ir fibers (brown) and LH-ir cells (blue) were distinguishable from each other in the pituitary. Although axonal routes of GnRH1-ir fibers to the pituitary were not evident, GnRH1-ir fibers were in close contact with LH-ir cells in the pituitary (Fig. 13C, D). In addition, a few GnRH3-ir fibers were in close contact with LH-ir cells in the pituitary (Fig. 13E, F).

Discussion

Here, we identified GnRH1 in the genome of the self-fertilizing fish, *K. marmoratus*, by *in silico* cloning. The distribution of GnRH1-ir, GnRH2-ir and GnRH3-ir cell bodies and fibers in the brain and pituitary of the *K. marmoratus* was determined by immunohistochemistry. The distribution of *gnrh1*-, *gnrh2*- and *gnrh3*-expressing neurons in the brain was determined by *in situ* hybridization. Moreover, the interaction between GnRH and LH in the pituitary was revealed by double immunohistochemistry.

GnRH1 neurons were detected in the ventral part of the PO by immunohistochemistry analysis and *in situ* hybridization. GnRH1-ir fibers were observed throughout the brain. Although the axonal routes of GnRH1-ir fibers to the pituitary were not clear, some of these fibers were in close contact with LH-ir cells in the pituitary as in the case of the Japanese flounder *Paralichthys olivaceus*, (Pham et al., 2007) and chub mackerel *Scomber japonicas* (Selvaraj et al., 2009). These results indicate that GnRH1 is responsible for gonadal maturation through LH secretion in *K. marmoratus*. In the barfin flounder *Verasper moseri* and the Japanese flounder, GnRH1 (seabream GnRH)-ir fibers were mainly localized in the preoptic area-hypothalamus-pituitary region, which formed a distinctive bundle of axons projecting to the pituitary, and were not distributed in other areas of the brain (Amano et al., 2002; Pham et al., 2007). However, in the case of *K. marmoratus*, GnRH1-ir fibers were distributed not only in the preoptic area-hypothalamus-pituitary region, but also in the other regions of the brain. These results suggest that GnRH1 functions as a neuromodulator in the brain possibly by compensating for the functions of GnRH2 and GnRH3. Unlike LH, no FSH-ir cells were detected in the pituitary. To our knowledge, this is the first study to immunostain FSH-ir cells in the pituitary of *K. marmoratus* using antibody raised against mummichog FSHβ 50-60 (Lot. 003). Thus, we cannot compare the present results with previously reported results. Although more precise study of FSH-ir cells is necessary, FSH immunoreactivity may change according to reproductive stages.

GnRH2 neurons were detected in the MT by both histological methods, and GnRH2-ir fibers were observed throughout the brain but not in the pituitary. These results indicate that GnRH2 does not regulate GTH secretion directly, but GnRH2 can be a neuromodulator and influence GTH in other ways. Although GnRH2-ir cell bodies were detected in the dorsal part of the PO as in the case of bluefin tuna *Thunnus thynnus* (Palmieri et al., 2008), *gnrh2*-expressing neurons were not detected in this region by *in situ* hybridization. Because the localization of GnRH1-ir cell bodies and that of GnRH2-ir cell bodies in the PO were different, and GnRH3-ir cell bodies were not detected in the PO, the GnRH2-ir cell bodies detected in the dorsal part of the PO were not the result of a cross-reaction between GnRH1 and GnRH3. The observed staining pattern may be a result of a cross-reaction of the antibody with an unknown substance. Another possibility is that the expression of *gnrh2* in the dorsal part of the PO is too low to detect by *in situ* hybridization.

GnRH2 stimulates the reproductive behavior of female goldfish *Carassius auratus* (Volkoff and Peter, 1999) and inhibits food intake in goldfish (Matsuda et al., 2008; Kang et al., 2011) and zebrafish *Danio rerio* (Nishiguchi et al., 2012). Furthermore, GnRH2 is suggested to be involved in GTH secretion in goldfish because GnRH2-ir cell bodies have been detected in the hypothalamus, and both GnRH3-ir and GnRH2-ir fibers have been detected in the pituitary (Kim et al., 1995). In addition to GnRH3, GnRH2-ir fibers are also detected in the pituitary of a transgenic zebrafish line and nutrition-related processes and behaviors may be regulated by GnRH2 (Xia et al., 2014). In *K. marmoratus*, the function of GnRH2 needs to be clarified.

GnRH3 neurons were detected in the Vl by both histological methods, and GnRH3 neurons may correspond to cells of the terminal nerve ganglion. GnRH3-ir fibers were abundant in the hypothalamus, and a small number were detected in the pituitary. Moreover, some GnRH3-ir fibers were in close contact with LH-ir cells in the pituitary. These results indicate that in addition to GnRH1, GnRH3 is responsible for gonadal maturation through LH secretion in *K. marmoratus*. Incidentally, GnRH3-ir cell bodies were detected not only in the Vl but also in the NLT by immunohistochemistry, and *gnrh3*-expressing neurons were detected in the Vl and VM by *in situ*

hybridization. This discrepancy may be explained by the balance of synthesis, release, and degradation of GnRH3; the cell bodies detected by immunohistochemistry reflect a summation of the synthesis, release, and degradation of GnRH.

In general, in fish that have three forms of GnRH (GnRH1, GnRH2, and GnRH3), the GnRH3-ir cell bodies are located in the terminal nerve ganglion and are not involved in GTH secretion (Okubo and Nagahama, 2008). However, in medaka, *gnrh3*-expressing neurons are detected in the ventral telencephalon, preoptic area, and anterior mesencephalon (Okubo et al., 2006). In some teleost fish that have lost GnRH1, such as masu salmon *Oncorhynchus masou* and goldfish, GnRH3-ir cell bodies are located in the preoptic area and hypothalamus and are involved in GTH secretion (Amano et al., 1991; Kim et al., 1995). Furthermore, in some teleost fish that have lost GnRH3, such as the European eel *Anguilla anguilla* (Montero et al., 1994) and the African catfish *Clarias gariepinus* (Zandbergen et al., 1995; Dubois et al., 2001), GnRH1-ir cell bodies are located not only in the preoptic area and hypothalamus but also in the terminal nerve, and are involved in GTH secretion. Taken together, we hypothesize that in *K. marmoratus* GnRH3 and GnRH1 are involved in LH secretion as has been reported for the European sea bass *Dicentrarchus labrax* (González-Martínez et al., 2001, 2002). This may be a convenient way to facilitate self-fertilization, but more precise studies are needed to further elucidate this mechanism.

In *K. marmoratus*, the GnRHR gene was identified, and its expression was examined (Rhee et al., 2008). GnRHR gene expression was predominantly observed in the brain, pituitary, and gonad. Hermaphrodite fish and secondary male fish have similar GnRHR gene expression patterns in tissues with higher expression levels in hermaphrodite fish. GnRHR gene expression increased from 2 days post-fertilization (dpf) to 12 dpf and decreased at the hatching stage when germ cells had started the oogenesis (Soto et al., 1992; Cole and Noakes, 1997; Kanamori et al., 2006; Minamimoto et al., 2006). Rhee et al. (2008) suggested that in *K. marmoratus*, the GnRHR gene has an important role in the transition of reproductive systems from hermaphrodite fish to secondary males. It will be interesting to clarify the relationship between GnRH and GnRHR levels

in the brain of *K. marmoratus*.

In conclusion, it is indicated that GnRH1 and possibly GnRH3 are responsible for the neural control of the reproductive endocrinology in *K. marmoratus*. Because of the wide distribution of GnRH1-ir fibers and GnRH3-ir fibers in the brain, it is suggested that not only GnRH2 but also GnRH1 and GnRH3 function as neurotransmitters or neuromodulators in the brain.

Acknowledgements

We thank Dr. Akio Shimizu for a gift of universal antisera raised against mummichog FSHβ and LHβ.

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393

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Figure legends

Fig. 1. *In silico* cloning of *K. marmoratus* GnRHs. (A) Nucleotide and deduced amino acid sequence of the *K. marmoratus* GnRH1 gene. The mature GnRH1 peptide sequence is shaded in gray. The asterisk indicates the stop codon. The polyA signal is underlined. (B) Alignments of the deduced amino acid sequences of GnRH1, GnRH2, and GnRH3 precursors in *K. marmoratus* with those in mummichog and medaka. Identical amino acids in all sequences are shaded in gray. The mature GnRH1 peptide sequence is underlined.

Fig. 2. Phylogenetic analysis of GnRHs in *K. marmoratus* and other teleosts. The number at each node indicates bootstrap values for 1000 replicates. The scale bar represents 0.1 substitutions per site.

Fig. 3. (A) Schematic illustration of the distribution of GnRH1-ir cell bodies (closed circles) in the ventral part of the PO and GnRH1-ir fibers (lines) in a parasagittal section. (B) Schematic illustration of the distribution of GnRH1-ir fibers in a midsagittal section. Left is rostral. Bar indicates 500 μm. C cerebellum, Hyp hypothalamus, M medulla oblongata, OB olfactory bulb, ON optic nerve, OT optic tectum, Pit pituitary, T telencephalon.

Fig. 4. Schematic drawing of the distribution of GnRH1-ir cell bodies (closed circles) and fibers (lines) in a frontal section from olfactory bulbs (A) to cerebellum/medulla oblongata (F). Top is dorsal. Bar indicates 200 μm.

Fig. 5. Immunohistochemistry images for GnRH1. (A) Sagittal section through the telencephalon and hypothalamus. (B) Higher magnification of boxed area in (A). GnRH1-ir cell bodies (arrowheads) in the ventral part of the PO and GnRH1-ir fibers are observed. (C) Adjacent section

of (B). No GnRH1-ir cell bodies and fibers are observed when anti-GnRH1 antibody was pre-absorbed overnight at 4 °C with an excess amount of synthetic GnRH1. (D) Sagittal section through the telencephalon. GnRH1-ir cell bodies and fibers do not disappear when anti-GnRH1 antibody was pre-absorbed overnight at 4 °C with an excess amount of KLH. (E) Frontal section through the telencephalon. (F) Higher magnification of boxed area in (E). GnRH1-ir cell bodies in the ventral part of the PO (arrowheads) and fibers are observed. (G) Sagittal section through the telencephalon. GnRH1-ir cell bodies in the Vl (arrowheads) are observed. (H) Sagittal section through the pituitary. (I) Higher magnification of boxed area in (H). GnRH1-ir fibers (arrowheads) are observed in the NH. (J) Adjacent section of (H). (K) Higher magnification of boxed area in (J). No GnRH1-ir fibers are observed but non-fibrous staining in the RPD, PPD and PI (white asterisks) remain when anti-GnRH1 antibody was pre-absorbed overnight at 4 °C with an excess amount of synthetic GnRH1. (L) Frontal section through the pituitary. (M) Higher magnification of boxed area in (L). GnRH1-ir fibers (arrowheads) are observed in the NH. Left is rostral for the sagittal section and top is dorsal for the frontal section. Bars indicate 100 μm (A, D, F, H, J, L) and 20 μm (B, C, E, G, I, K, M). NH neurohypophysis, OT optic tectum, Pit pituitary, PI pars intermedia, PO preoptic nucleus, PPD proximal pars distalis, RPD rostral pars distalis, T telencephalon, Vl lateral part of ventral telencephalic area.

Fig. 6. (A) Schematic illustration of the distribution of GnRH2-ir cell bodies (closed circles) in the MT and GnRH2-ir fibers (lines) in a parasagittal section. (B) Schematic illustration of the distribution of GnRH2-ir cell bodies in the dorsal part of the PO (closed circles) and GnRH2-ir fibers (lines) in a midsagittal section. Staining of the pituitary is shown in gray. Left is rostral. Bar indicates 500 μm. C cerebellum, Hyp hypothalamus, M medulla oblongata, OB olfactory bulb, ON optic nerve, OT optic tectum, Pit pituitary, T telencephalon.

Fig. 7. Schematic drawing of the distribution of GnRH2-ir cell bodies (closed circles) and fibers

559 (lines) in a frontal section from olfactory bulbs (A) to cerebellum/medulla oblongata (F). Top is
560 dorsal. Bar indicates 200 μm.
561
562 Fig. 8. Immunohistochemistry images for GnRH2. (A) Sagittal section through the MT. Dense
563 staining is detected in the PI of the pituitary (arrowhead). (B) Higher magnification of boxed area in
564 (A). GnRH2-ir cell bodies (arrowheads) in the MT and GnRH2-ir fibers are observed. (C) Frontal
565 section through the MT. (D) Higher magnification of boxed area in (C). GnRH2-ir cell bodies
566 (arrowheads) and GnRH2-ir fibers are observed. (E) Frontal section through the diencephalon. (F)
567 Higher magnification of boxed area in (E). GnRH2-ir cell bodies (arrowheads) in the dorsal part of
568 PO and GnRH2-ir fibers are observed. (G) Sagittal section through the MT. GnRH2-ir cell bodies
569 (arrowheads) and fibers are observed. (H) Adjacent section of (G). No GnRH2-ir cell bodies and
570 fibers are observed when the anti-GnRH2 antibody was pre-absorbed overnight at 4 °C with an
571 excess amount of synthetic GnRH2. Left is rostral for the sagittal section and top is dorsal for the
572 frontal section. Bars indicate 100 μm (A, C, E) and 20 μm (B, D, F, G, H). MT midbrain tegmentum,
573 OT optic tectum, Pit pituitary, PO preoptic nucleus, T telencephalon.
574
575 Fig. 9. (A) Schematic illustration of the distribution of GnRH3-ir cell bodies (closed circles) in the
576 Vl and GnRH3-ir fibers (lines) in a parasagittal section. (B) Schematic illustration of the
577 distribution of GnRH3-ir cell bodies in the NLT (closed circles) and GnRH3-ir fibers (lines) in a
578 midsagittal section. Although GnRH3-ir fibers are not shown in the pituitary in this section,
579 GnRH3-ir fibers are detected in an adjacent section (see Fig. 10F). Left is rostral. Bar indicates 500
580 μm. C cerebellum, Hyp hypothalamus, M medulla oblongata, OB olfactory bulb, ON optic nerve,
581 OT optic tectum, Pit pituitary, T telencephalon.
582
583 Fig. 10. Schematic drawing of the distribution of GnRH3-ir cell bodies (closed circles) and fibers
584 (lines) in a frontal section from olfactory bulbs (A) to cerebellum/medulla oblongata (G). Top is

585 dorsal. Bar indicates 200 μm.

586

587 Fig. 11. Immunohistochemistry images for GnRH3. (A) Frontal section through the telencephalon.
588 (B) Higher magnification of boxed area in (A). GnRH3-ir cell bodies (arrowheads) in the Vl and
589 GnRH3-ir fibers are observed. (C) Sagittal section through the hypothalamus. (D) Higher
590 magnification of boxed area in (C). GnRH3-ir cell body (arrowhead) in the NLT and GnRH3-ir
591 fibers are observed. (E) Sagittal section through the hypothalamus and pituitary. (F) Higher
592 magnification of boxed area in (E). GnRH3-ir fibers are abundant in the hypothalamus (arrowheads)
593 and only a few GnRH3-ir fibers are detected in the pituitary (double arrowhead). (G) Sagittal
594 section through the hypothalamus. GnRH3-ir fibers are observed (arrowheads). (H) Adjacent
595 section of (G). No GnRH3-ir fibers are observed when anti-GnRH3 antibody was pre-absorbed
596 overnight at 4 °C with an excess amount of synthetic GnRH3. Left is rostral for the sagittal section
597 and top is dorsal for the frontal section. Bars indicate 100 μm (A, C, E) and 20 μm (B, D, F, G, H).
598 Hyp hypothalamus, NLT lateral tuberal nucleus, Pit pituitary, Vl lateral part of ventral telencephalic
599 area.

600

601 Fig. 12. (A) Sagittal section through the telencephalon, hypothalamus and pituitary showing *in situ*
602 hybridization for *GnRH1*. No *GnRH1*-expressing cells are observed in the pituitary (arrowhead).
603 (B) Higher magnification of boxed area in (A). *GnRH1*-expressing neurons (arrowheads) are
604 detected in the ventral part of the PO. (C) Sagittal section through the MT and pituitary showing *in*
605 *situ* hybridization for *GnRH2*. No *GnRH2*-expressing cells are observed in the pituitary (arrowhead).
606 (D) Higher magnification of boxed area in (C). *GnRH2*-expressing neurons (arrowheads) are
607 detected in the MT. (E) Sagittal section through the telencephalon and hypothalamus showing *in*
608 *situ* hybridization for *GnRH3*. (F) Higher magnification of boxed area (left) in (E).
609 *GnRH3*-expressing neurons (arrowheads) are detected in the Vl. (G) Higher magnification of boxed
610 area (right) in (E). *GnRH3*-expressing neurons (arrowheads) are detected in the VM. Left is rostral.

611 Bars indicate 100 μm (A, C, E) and 20 μm (B, D, F, G). MT midbrain tegmentum, Pit pituitary, PO
612 preoptic nucleus, Vl lateral part of ventral telencephalic area, VM ventromedial thalamic nucleus.
613
614 Fig. 13. (A) Sagittal section through the pituitary. LH-ir cells are observed in the pituitary
615 (arrowheads). (B) Adjacent section of (A). No LH-ir cells are observed when the anti-LHβ antibody
616 was pre-absorbed overnight at 4 °C with an excess amount of synthetic LHβ. (C) Image showing
617 double immunohistochemistry for GnRH1 and LH. Sagittal section through the pituitary. (D)
618 Higher magnification of boxed area in (C). GnRH1-ir fibers (brown, arrowheads) are in close
619 contact with LH-ir cells (blue, asterisks). (E) Image showing double immunohistochemistry for
620 GnRH3 and LH. Sagittal section through the pituitary. (F) Higher magnification of boxed area in
621 (E). GnRH3-ir fibers (brown, arrowheads) are in close contact with LH-ir cells (blue, asterisks).
622 Left is rostral. Bars indicate 100 μm (A, B, C, E) and 20 μm (D, F).
623

Abbreviations

CE	cerebellar corpus
cre	cerebellar crest
D	dorsal telencephalic area
Dc	central part of dorsal telencephalic area
Dd	dorsal part of dorsal telencephalic area
dDm	dorsal region of Dm
Dl	lateral part of dorsal telencephalic area
Dm	medial part of dorsal telencephalic area
Dp	posterior part of dorsal telencephalic area
DT	dorsal thalamus
E	entopeduncular nucleus
EG	granular eminence
GL	granular layer
GR	corpus glomerulosum pars rotunda
MC	Mauthner's cell
NFLM	nucleus of the medial longitudinal fascicle
NLT	lateral tuberal nucleus
NM	medial nucleus of rhombencephalic octavolateral area
NRL	nucleus of lateral recess
OB	olfactory bulb
OT	optic tectum
PCL	Purkinje cell layer
PGm	medial preglomerular nucleus
PGZ	periventricular gray zone of optic tectum

650	Pit	pituitary
651	PO	preoptic nucleus
652	PTh	prethalamic nucleus
653	RF	reticular formation
654	SCN	suprachiasmatic nucleus
655	TL	longitudinal torus
656	TS	semicircular torus
657	Vc	central part of ventral telencephalic area
658	Vd	dorsal part of ventral telencephalic area
659	vDm	ventral region of Dm
660	vem	mesencephalic ventricle
661	ver	rhombencephalic ventricle
662	Vl	lateral part of ventral telencephalic area
663	VM	ventromedial thalamic nucleus
664	Vp	posterior part of ventral telencephalic area
665	Vv	ventral part of ventral telencephalic area

A

K. marmoratus GnRH1

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AATGGCTGTAAAACCCTTGACTGTGTGGCTGCTGCTGGTGGGGACGCTGCTGCCGCTGGGCTGCTGTCAGCAC
  M  A  V  K  P  L  T  V  W  L  L  L  V  G  T  L  L  P  L  G  C  C  Q  H
TGGTCGTTCGGTCTGAGCCCAGGAGGGAAGAGAGAACTGGACGGCTTGGCGAACACACTGCAAGACgtcagta
  W  S  F  G  L  S  P  G  G  K  R  E  L  D  G  L  A  N  T  L  Q  D
cttttctcagaacacgtctcgcttcttttaaaattgactgctcctcatatgtttagctgatattgtccttttt
ttttaaattttaatttaatttttgtttgtttcttgttacagATAGTTGAGGGGTTTGCACACATGGATGCACCT
                                                 I  V  E  G  F  A  H  M  D  A  P
TGCAGAGTTCTGAGTTGTGAAGAGGAATCCCCTTTTGCTAAATTGTACAGAGTGAAGGGGCTTCTTgtaagtc
  C  R  V  L  S  C  E  E  E  S  P  F  A  K  L  Y  R  V  K  G  L  L
tgttgatccttttttttttttatttgctgttttcgccactcatttgtcataaaagtcaaatgcagcgtggtgc
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ttagagaacaaattactgaggcgggtgaatacttctgcagcccactgtgagttatgaggttctgataaataca
aaaaatgaactggactcacatttctctcctcttcttcttttcagGGGAGCGTAACCGACAAGGAAGTTGGACA
                                                 G  S  V  T  D  K  E  V  G  H
TCGAGGATACAAAAAATAATGTTTGATAAACTACTACATGCATGTCATGACTTGTATATCCAGATCACAAATA
R  G  Y  K  K  *
AAAGC
```

B

GnRH1

```
K. marmoratus   MAVKPLTVWLLLVG--TLLPLGCCQHWSFGLSPGGKRELDGLANTLQDIVEGFAHMDAPC
Mummichog       MAVKTLSLWLLLAWTVGLLSLGSCQHWSFGLSPGGKRELDVSPDRLDSIFEGLAHVGAPC
Medaka          MVVKTWMPWLLVSS---VLSQGCCQHWSFGLSPGGKRELKYFPNTLENQIR-LLNSNTPC

K. marmoratus   RVLSCEEESPFAKLYRVKGLLGSVTDKEVGHRGYKK
Mummichog       SVPGCAEESPFAKIHRLKGLLVRVHEREHGHQALKQ
Medaka          SDLSHLEESSLAKIYRIKGLLGSVTEAKNGYRTYK-
```

GnRH2

```
K. marmoratus   MMSRLILLLGLLLYVGAQLSSAQHWSHGWYPGGKRELDSYGAPEISEDIKLCEPGECSYL
Mummichog       -MSRLVLLLVLFVCVGAQLCYAQHWSHGWYPGGKRELDSFGASEMSEEVKLCEAGECSYL
Medaka          -MSRLVLLLGVLLYVGAQLSQAQHWSHGWYPGGKRELNSF---EVSEEMKLCETGECSYM

K. marmoratus   RPQRRNILRDIVLDALARELQNRK
Mummichog       RPQRRSTLRNIVLDALARELQKRK
Medaka          RPQRRSFLRNIVLDALARELQKRK
```

GnRH3

```
K. marmoratus   MKANSRVMVQVLLLALVAQVTLCQHWSYGWLPGGKRSVGELEATIRMMGTGGVVSLPEEA
Mummichog       MKASNRVMVQVLLLALLAQVTLCQHWSYGWLPGGKRSVGELEATIRMMGTGGVVSLPEEA
Medaka          MDVSSKVVVQVLLLALVVQVTLCQHWSYGWLPGGKRSVGELEATIRMMGTGRVVSLPEDA

K. marmoratus   SAQTQERLRPYNIN-DDSSNFDRKVWKKTLE
Mummichog       SAQTQERLRPYHIN-DGSSHFDRNKRFLNE-
Medaka          SAQTQERLRQYNLINDGSTYFDRKKRFMSQ-
```

Fig. 1

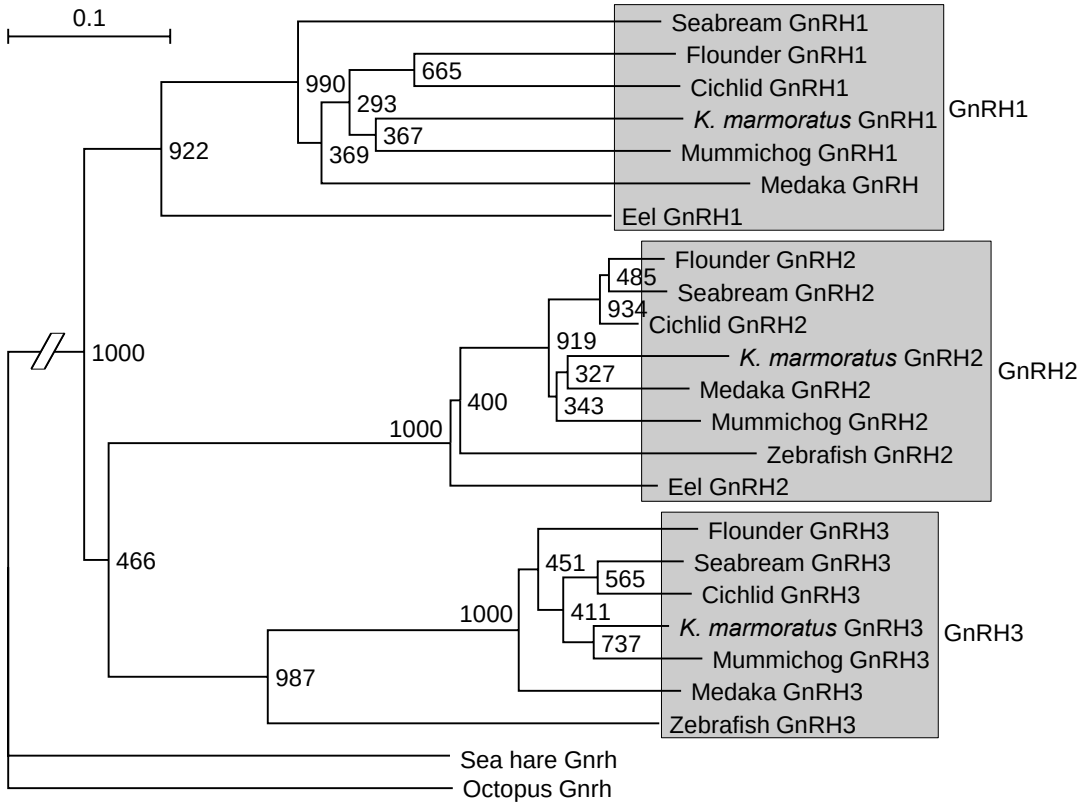
0.1
Seabream GnRH1
Flounder GnRH1
Cichlid GnRH1
K. marmoratus GnRH1
Mummichog GnRH1
Medaka GnRH
Eel GnRH1
GnRH1
665
990
293
367
369
922
Flounder GnRH2
485
Seabream GnRH2
934
Cichlid GnRH2
919
K. marmoratus GnRH2
327
Medaka GnRH2
343
Mummichog GnRH2
400
Zebrafish GnRH2
1000
Eel GnRH2
GnRH2
1000
Flounder GnRH3
451
Seabream GnRH3
565
Cichlid GnRH3
411
K. marmoratus GnRH3
737
Mummichog GnRH3
1000
Medaka GnRH3
987
Zebrafish GnRH3
GnRH3
466
Sea hare Gnrh
Octopus Gnrh

Fig. 2

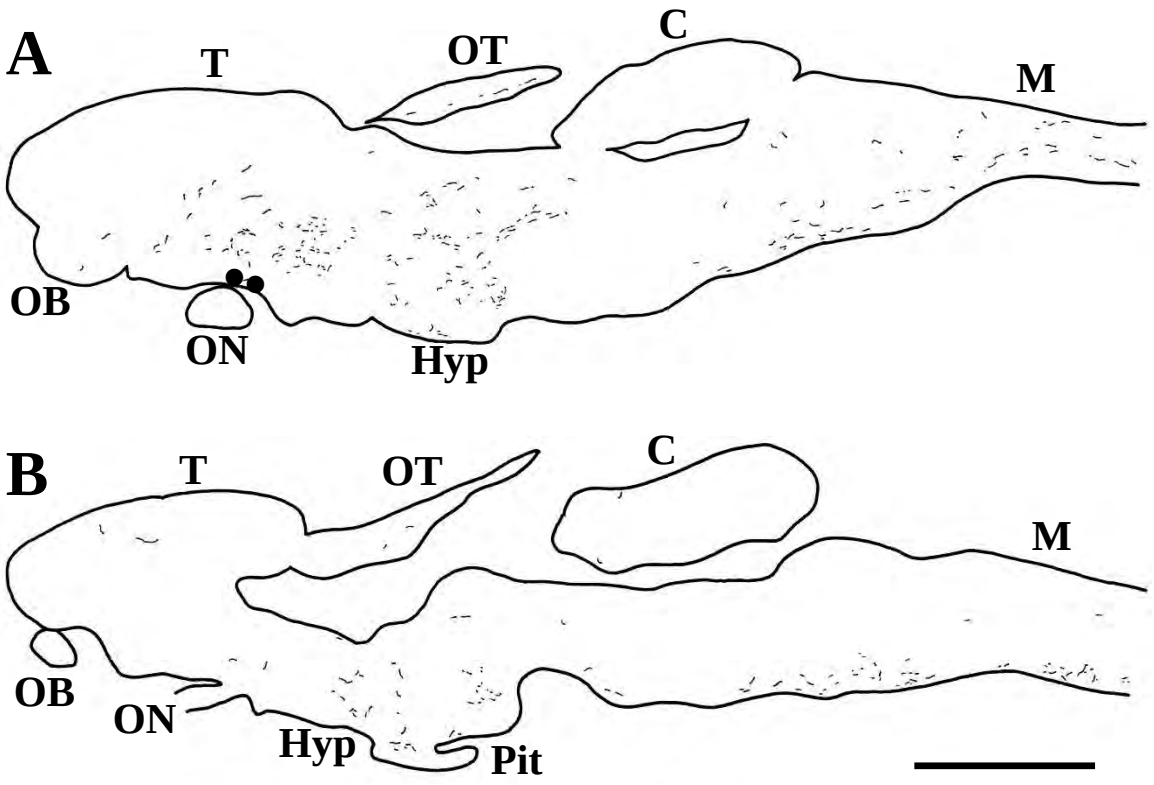

Fig. 3

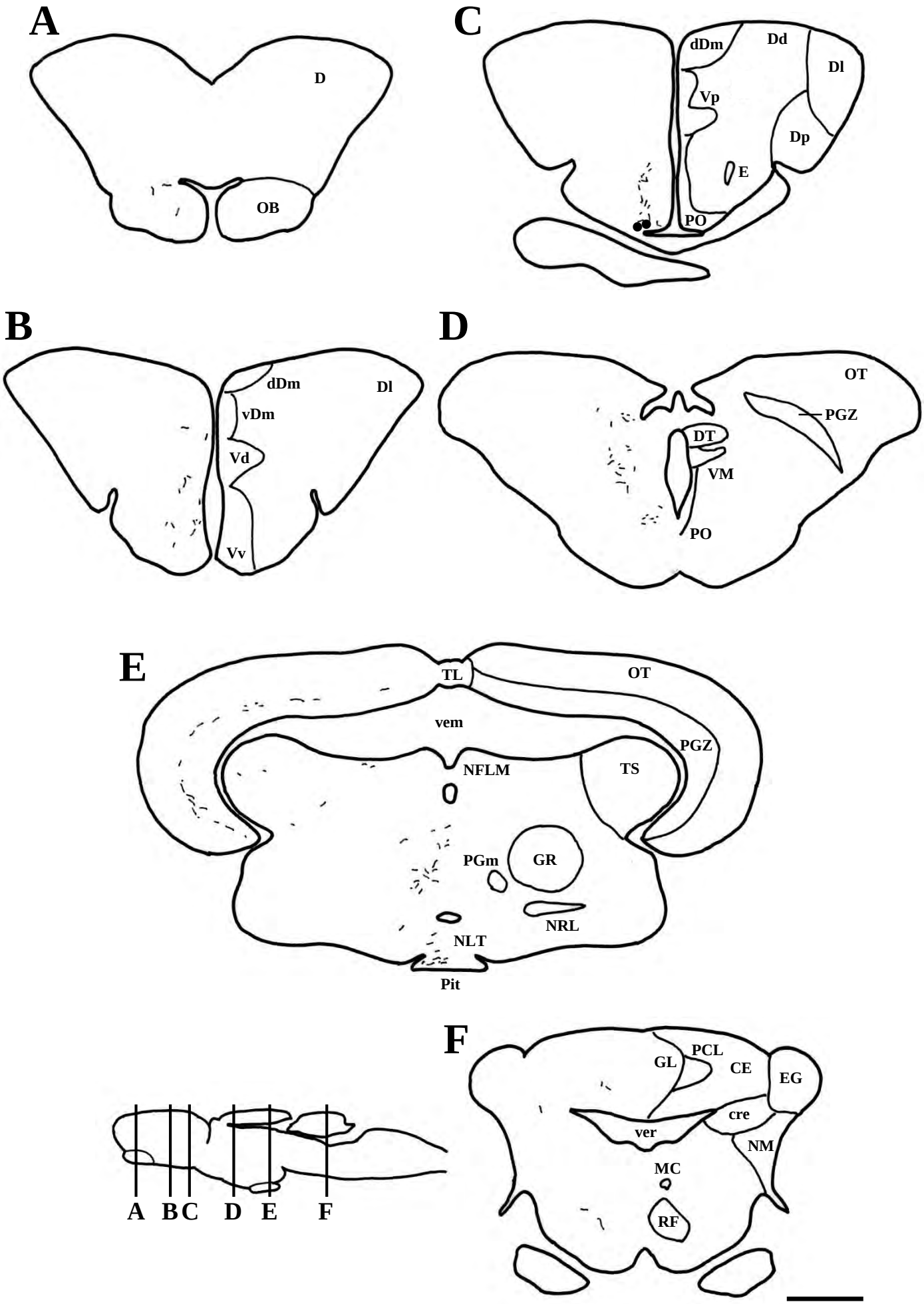
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Fig. 4

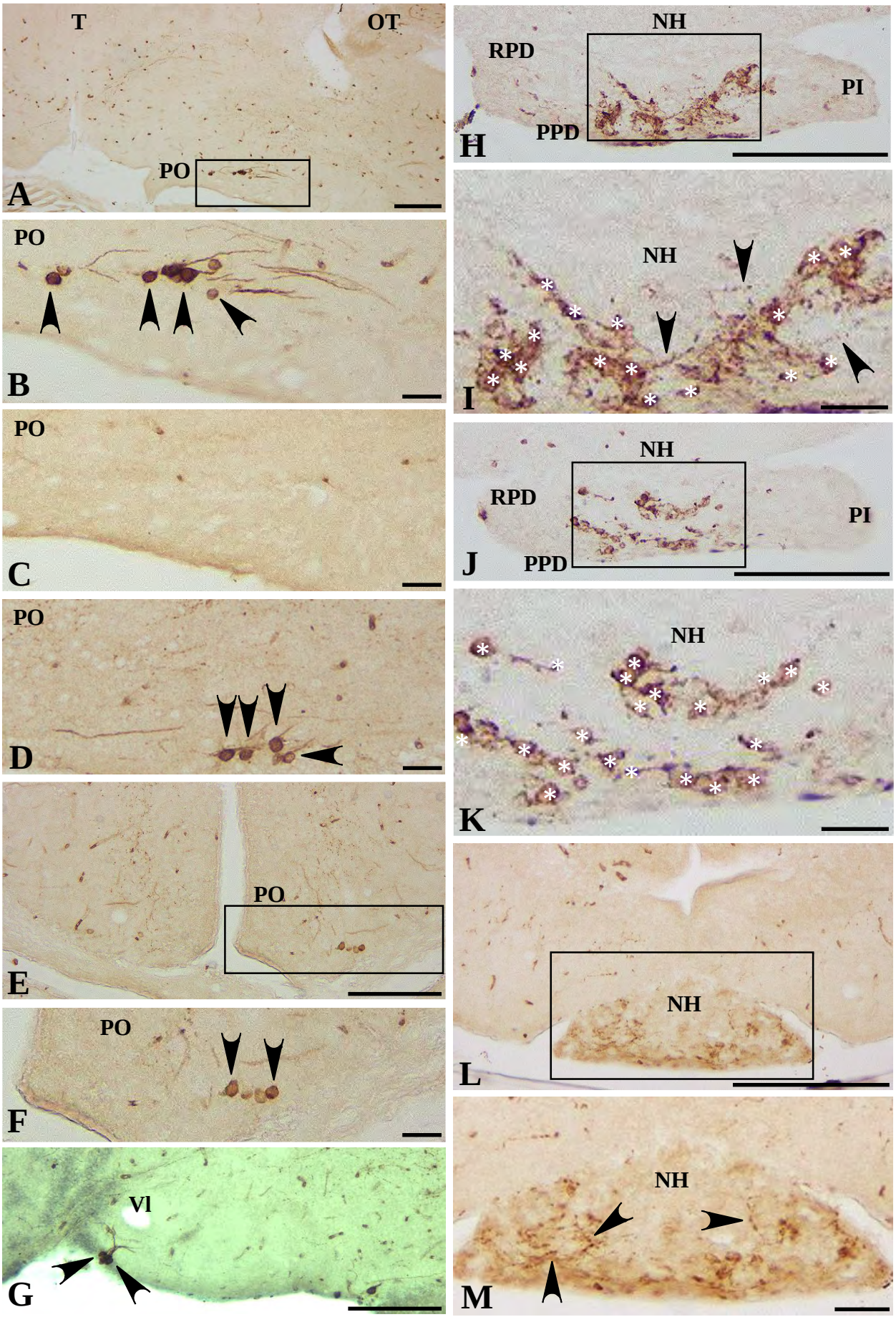
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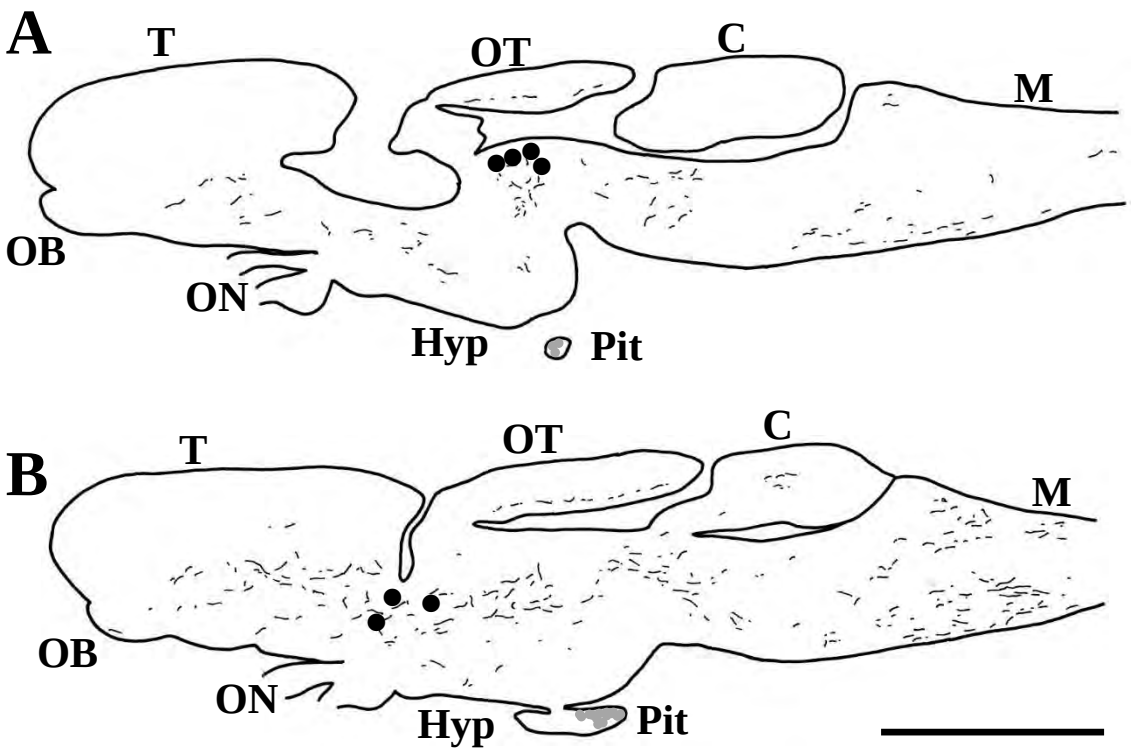
Fig. 5



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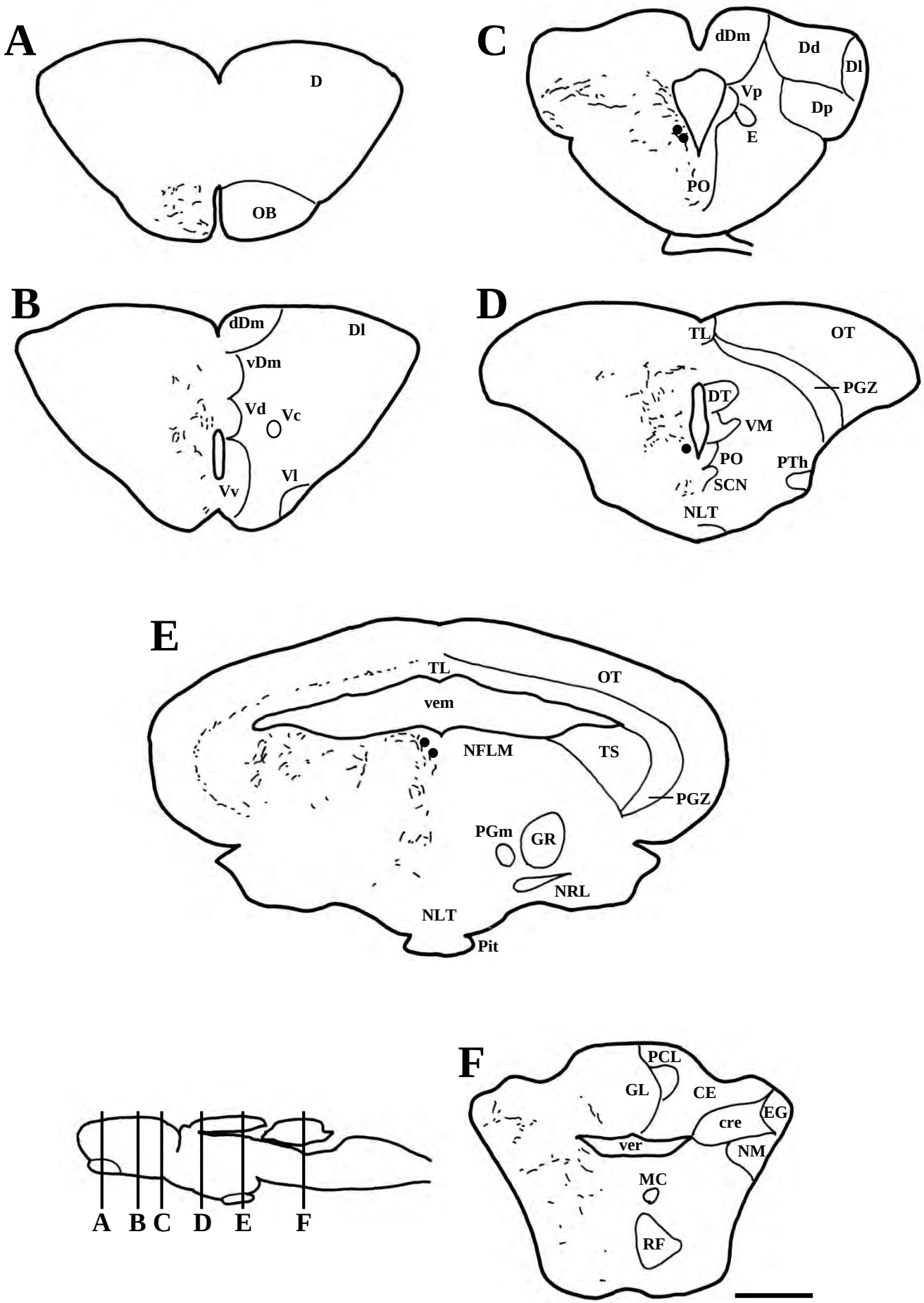
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Fig. 7

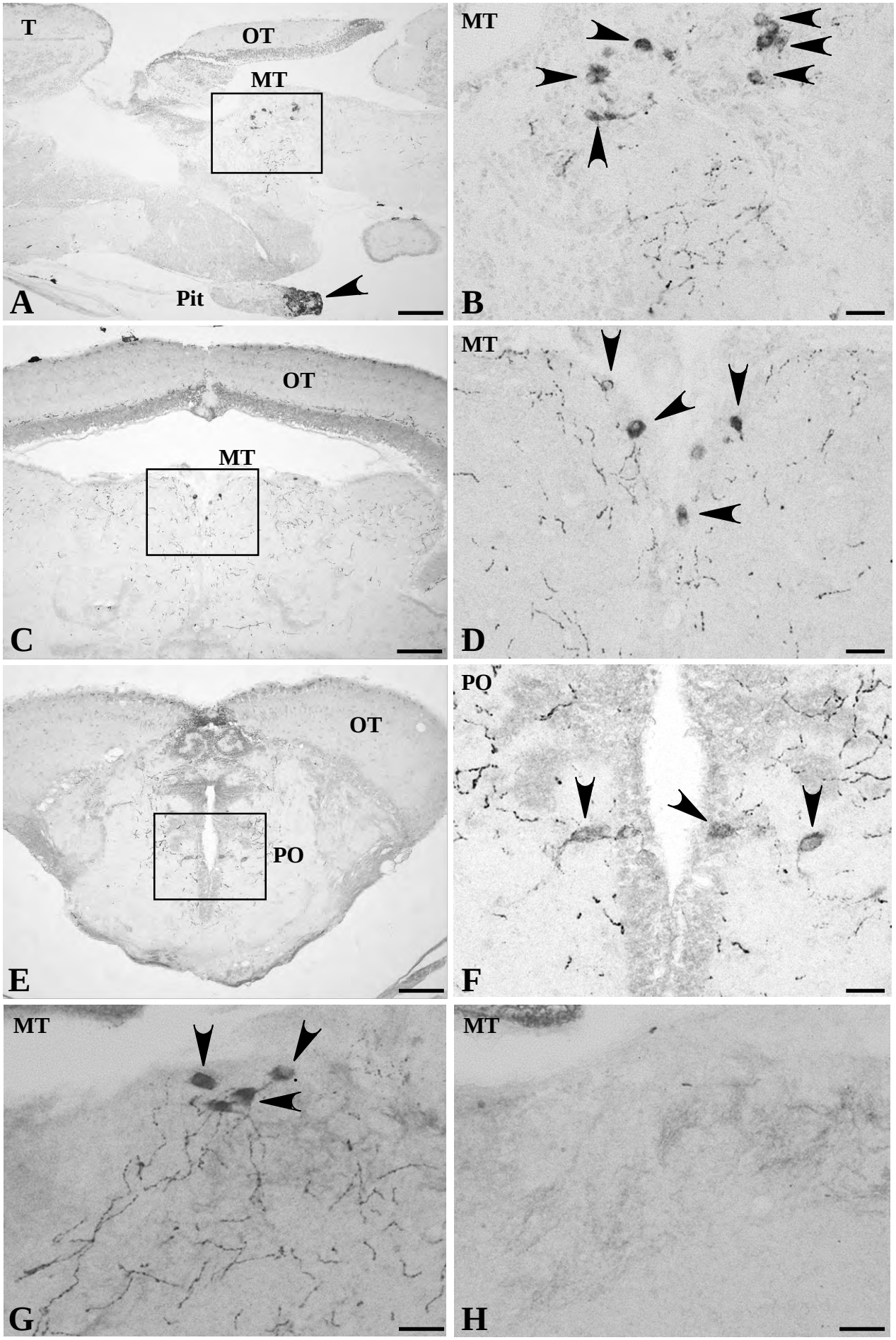

Fig. 8

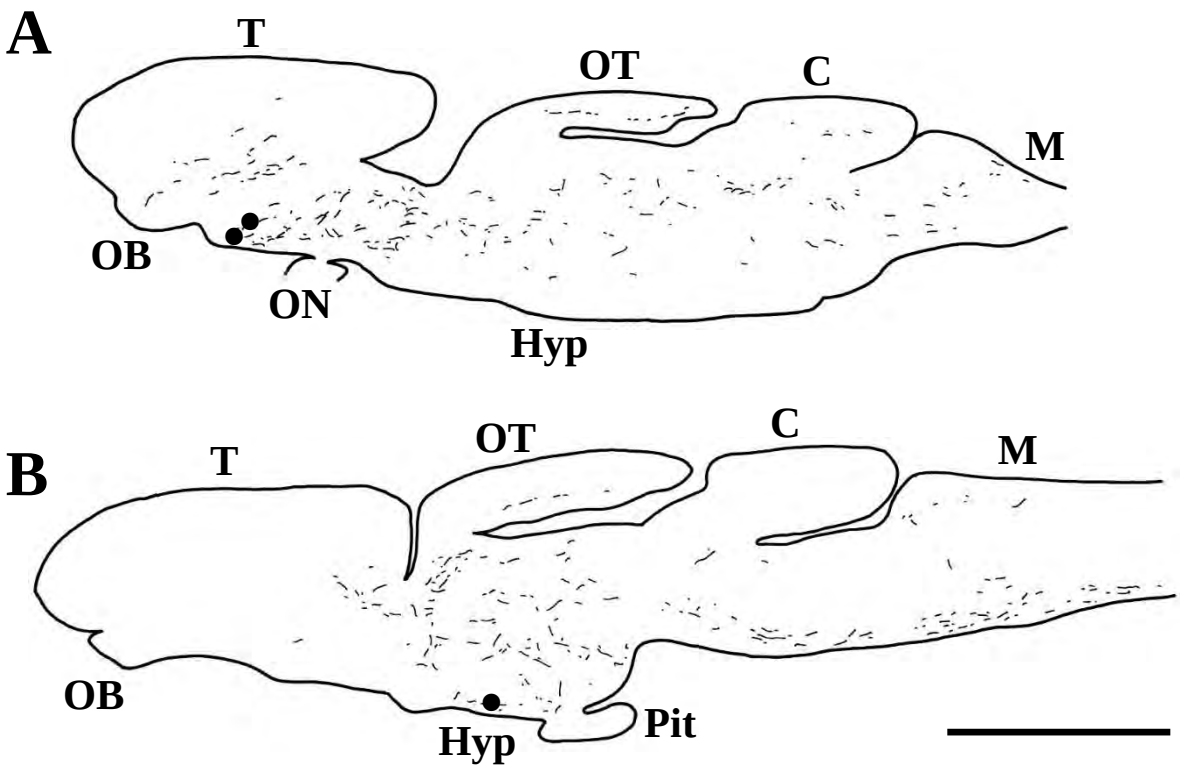

Fig. 9

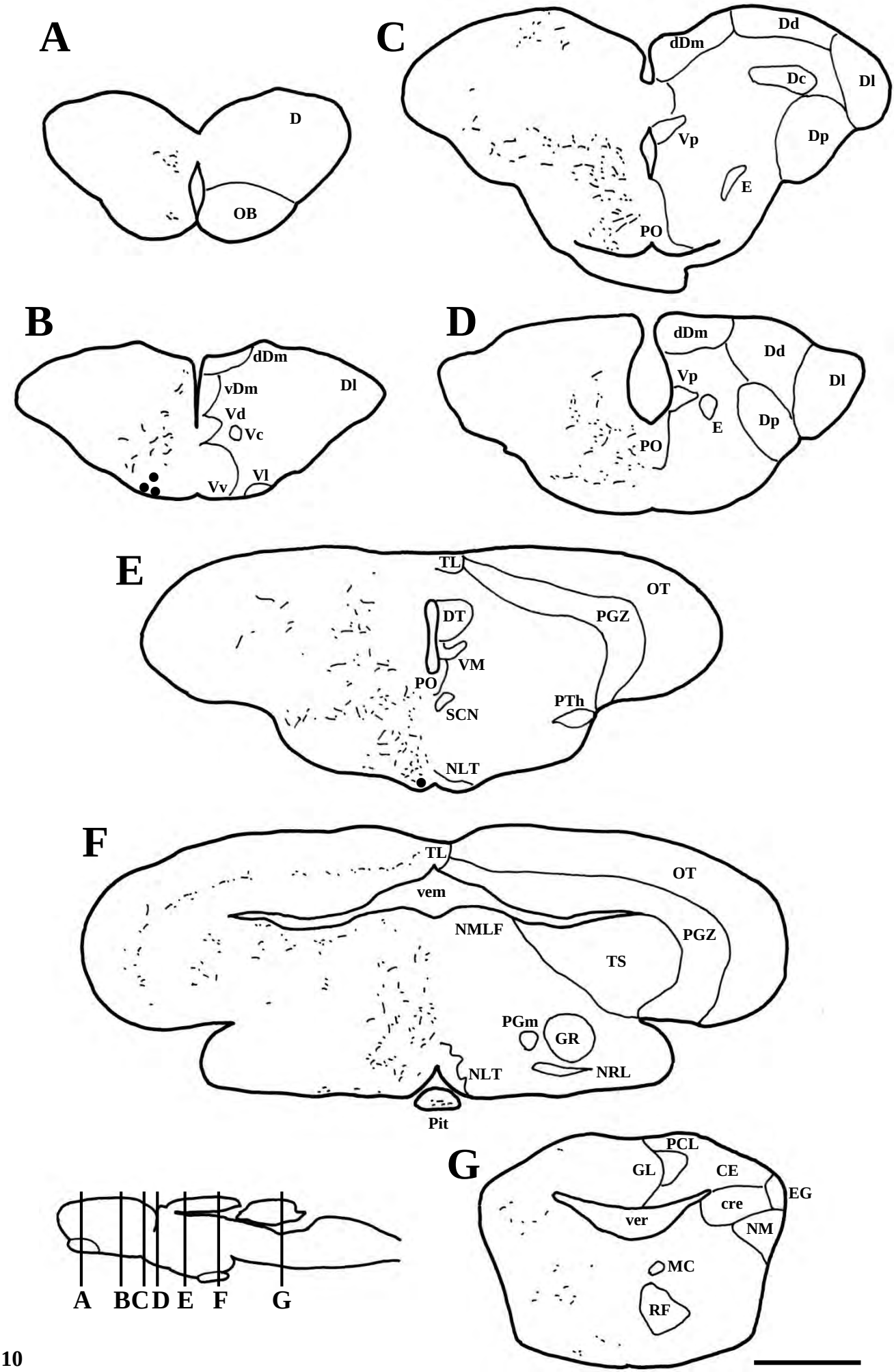
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Fig. 10

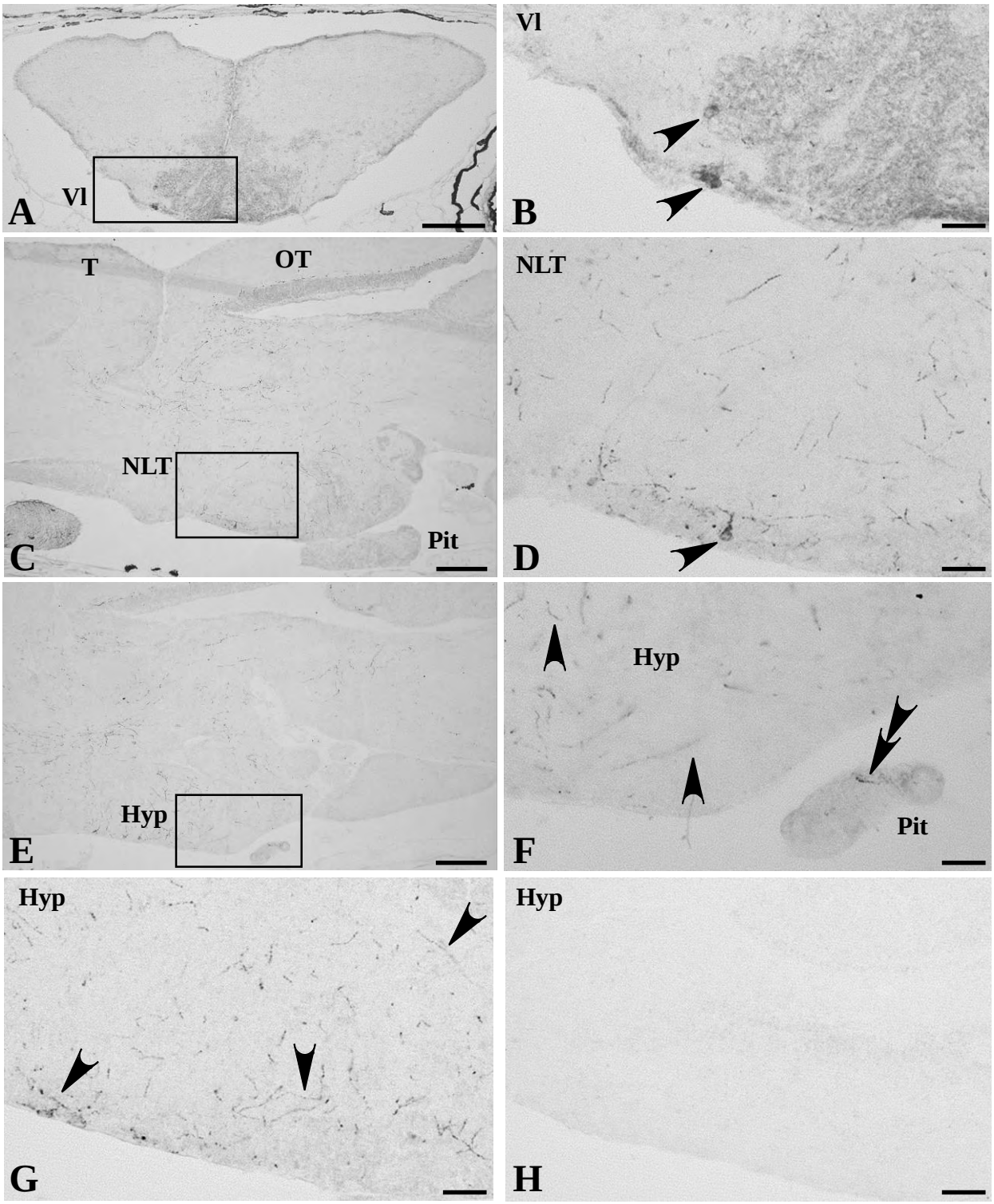

Fig. 11

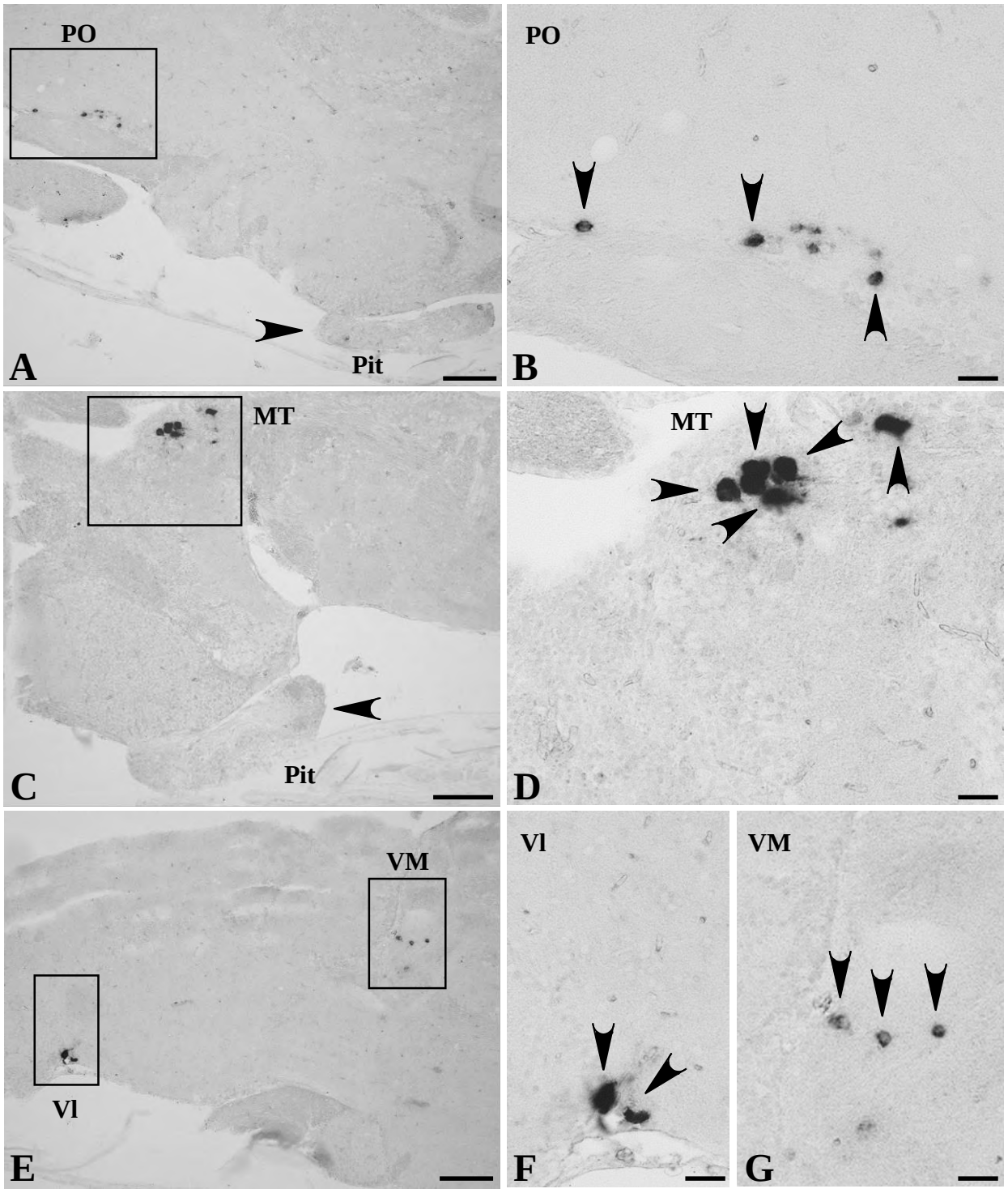

Fig. 12

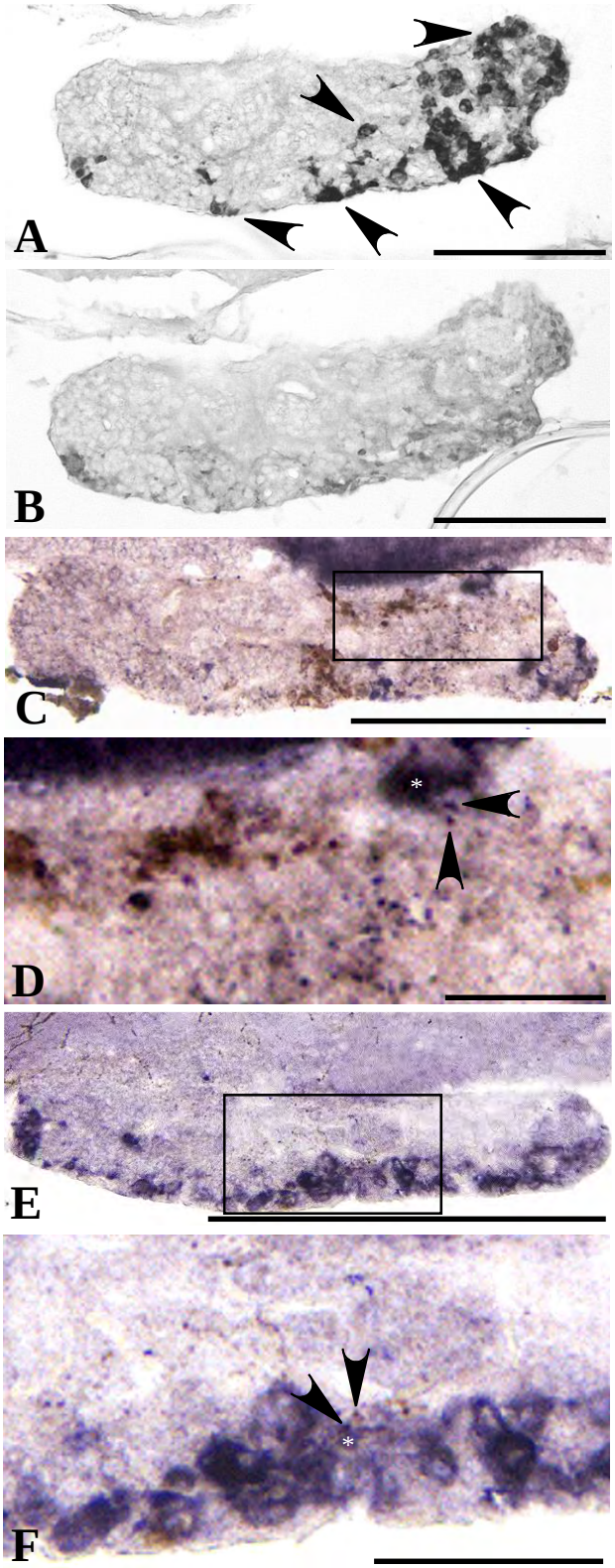

Fig. 13