Organization of Two Independent Kisspeptin Systems Derived from Evolutionary-Ancient Kiss Genes in the Brain of Zebrafish

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Kisspeptins are new actors in the neuroendocrine regulation of reproduction. In vertebrates, the number of *kiss* genes varies from none to three. Zebrafish have two *kiss* genes, *kiss1* and *kiss2*, and two kiss receptors (GPR54), *kiss1r* and *kiss2r*. To provide detailed information on the organization of the kiss systems in zebrafish, antibodies were raised against the C terminus of zebrafish preproKiss1 and preproKiss2. Immunohistochemistry fully confirmed *in situ* hybridization data, showing that *kiss1*-expressing neurons are only located in the habenular nucleus, while *kiss2*-expressing neurons are found in the dorsal and ventral hypothalamus. *Kiss1*-expressing cells project only to the interpeduncular and raphe nuclei and strongly expressed the *kiss1r* receptor. In contrast, *kiss2*-expressing cells are mostly present in the dorsal and ventral hypothalamus and project widely into the subpallium, the preoptic area, the thalamus, the ventral and caudal hypothalamus, and the mesencephalon. All these regions strongly expressed the *kiss2r* messengers. Kiss2 fibers profusely innervate the ventral forebrain and notably made close apposition with GnRH3 neurons. Estrogen treatment of juvenile fish with estradiol causes increase in *kiss2* and *kiss2r* expression. In the pituitary gland, no proKiss2-positive fibers were detected, while positive cells were observed in the pars intermedia. In addition to proposing a successful strategy to develop antibodies to kisspeptins, these data indicate that the *kiss2* systems of zebrafish are implicated in reproductive events, while the *kiss1* gene would play other functions that remain to be established. **(*Endocrinology* 152: 1527–1540, 2011)**

Before the discovery of kisspeptins, GnRH was acknowledged in all vertebrates as the major initiator of the hormonal cascade regulating the reproductive axis. Originally identified as a metastasis suppressor in mammals (1), the *kiss1* gene produces several peptides named kisspeptins (kisspeptin-54, -14, -13, -10), which activate the KISS1 receptor (KISS1R, GPR54) previously known as an orphan receptor (2). In 2003, kisspeptins emerged as key players in the regulation of the hypothalamic-pituitary-gonadal axis when KISS1R-null mutations were

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; GAP, GnRH-associated peptides; EF-1, elongation factor 1; HNPP, 2-hydroxy-3-naphtoic acid-2′-phenylanilide phosphate; IPN, interpeduncular nucleus; ir, immunoreactive; NBT/BCIP, nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt; SSC, standard saline citrate; TFA, trifluoroacetic acid.

found associated to idiopathic hypothalamic hypogonadism (3, 4). Nowadays, it is demonstrated that the KISS1 system plays essential roles in the neuroendocrine control of reproduction in mammals (5, 6). It is indeed considered that KISS1 neurons are major integrators of environmental (7), metabolic (8), and gonadal signals (9, 10). As such, they play key roles in the central triggering of puberty, acting upstream from GnRH to stimulate gonadotrophin release (11–13). In several mammalian species, the neuroanatomical localization of kisspeptin neurons and projections was reported either by *in situ* hybridization or immunohistochemistry (5, 14). The distribution of KISS1R-expressing cells was recently described in mouse (15).

Recent phylogenetic studies identified two kisspeptins and kisspeptin receptor genes in different vertebrate groups including fish, amphibians, and monotremes (16, 17). Notably, two kiss (termed *kiss1* and *kiss2*) and two kiss receptor (*kiss1r* and *kiss2r*) paralogous genes were reported in fish like lamprey, zebrafish, medaka, sea bass, and goldfish (16–22). Three genes for both ligands and receptors were described in amphibians (16). In mammals, although monotremes exhibit two genes, humans and rodents have only one (KISS1) and one receptor form KissR (16). The presence of two or three forms of kisspeptin and the cognate receptor genes in a single species of lower vertebrates supports the hypothesis of two rounds of genome duplication followed by degeneration and complementation of the genes (16). It would then be highly interesting to investigate how different Kiss-producing systems were originally organized and evolved in vertebrates.

Until now, information regarding localization of kisspeptins was hampered by the fact that obtaining specific antibodies is difficult because of the presence of an RF-amide sequence at the C terminus (14, 16), causing cross-reaction with peptides of the RFamide family. Current mammalian antibodies to KISS1–10 are useless in fish, despite the fact that they recognize fish Kiss1 (89–98) (YNLNSFGLRY-NH_2) and Kiss2 (83–92) (FNYNPFGLRF-NH_2) (16). Indeed, these antibodies cross-react with other peptidergic systems (A. Servili, A. Caraty, M. Carrillo, and O. Kah, unpublished data). Thus, to date, the neuroanatomical localization of Kiss-producing neurons in the brain of nonmammalian vertebrates, notably in fish, has been studied only at the mRNA level (16, 19, 20). In fish, the specific projections of the two kiss systems are unknown, and so are their potential interactions with other neuroendocrine circuits involved in the control of reproduction. Some quantitative PCR-based evidences suggest the role of the kiss systems on the onset of puberty and reproduction in fish (17–19, 23–27), but their interpretation remains difficult in the absence of detailed information on the neuroanatomical organization of these systems.

The aim of this work was then to obtain detailed information regarding the organization of the Kiss1 and Kiss2 systems in zebrafish brain. To this end we produced specific antibodies that are able to unambiguously distinguish zebrafish preproKiss1 from preproKiss2. Another aim was to map the *kiss1r* and *kiss2r* in relation to Kiss1 and Kiss2 neuronal projections and to document potential interactions between Kiss and GnRH systems. Finally, we aimed at looking at the effects of estradiol on *kiss1* and *kiss2* gene expression.

Materials and Methods

Animals

All zebrafish (*Danio rerio*) were originally purchased from the Tübingen zebrafish stock center and maintained in our local facility of the Institut Fédératif de Recherche (IFR) 140 [Institut National de la Recherche Agronomique-Station Commune de Recherches en Ichtyophysiologie, Biodiversité et Environnent (SCRIBE), Rennes]. They are raised in 28.5 C recirculated water and kept under a 14-hr dark/10-hr light cycle. Animals were handled in agreement with the European Union regulation concerning the use and protection of experimental animals. All procedures complied with the in-house rules of the Unité Mixte de Recherche (UMR) Centre National de la Recherche Scientifique (CNRS) 6026.

Riboprobe synthesis

Specific riboprobes were synthesized using pGEM-T Easy Vector (Promega, Charbonnieres, France) or pcDNA3 (Invitrogen, Gercy Pontoise, France), (as detailed in Table 1) containing cDNA of zebrafish *kiss1* (20), *kiss2*, *kiss1r*, and *kiss2r* (16). Antisense and sense single-stranded mRNA probes were obtained with DIG RNA labeling MIX (Roche, Indianapolis, IN) by transcription with T7 and SP6 polymerase (Promega, Madison, WI) on plasmids linearized by the *sacII* and *salI* or *XhoI* and *BamHI* restriction enzymes (see Table 1).

In situ hybridization

Serial transverse 12-μm-thick cryostat sections were mounted onto poly-L-Lysine-coated slides. The protocol for *in*

TABLE 1. Detailed information on the riboprobes synthesis for the genes studied

Gene	Vector	Size	Enzymes/polymerases for probe synthesis
Kiss1	pGEM-T Easy Vector	660 pb	Antisense: *sacII*/Sp6 Sense: *salI*/T7
Kiss2	pGEM-T Easy Vector	486 pb	Antisense: *sacII*/Sp6 Sense: *salI*/T7
Kiss1r	pcDNA3	1095 pb	Antisense: *BamHI*/Sp6 Sense: *XhoI*/T7
Kiss2r	pcDNA3	1083 pb	Antisense: *BamHI*/Sp6 Sense: *XhoI*/T7

situ hybridization was performed as previously described (55) with modifications. Before hybridization, sections were washed in 0.1 M PBS (pH 7.4) and postfixed for 20 min in 4% paraformaldehyde diluted in PBS. After washing (PBS 20 C), sections were treated with proteinase K for five minutes at room temperature [10 mg/ml in 50 mM Tris-HCl (pH 8.0), 5 mM EDTA], rinsed, and fixed in 4% paraformaldehyde. Sections were rinsed twice in 2 × standard saline citrate (SSC). Hybridization was performed at 65 C in a humidified chamber using 100 μl hybridization buffer (2 × SSC; 2.5% dextran sulfate; 50% deionized formamide; 5 × Denhardt's solution; 50 μg/ml of yeast tRNA, pH 8.0; 4 mM EDTA) containing the DIG-labeled probe (1–3 μg/ml). After hybridization, slides were rinsed in 2 × SSC at 65 C, followed by two rinses at 65 C (2 × 30 min) in 2 × SSC/50% formamide. Final rinses were made in 0.2 and 0.1 × SSC at room temperature, and sections were processed for immunodetection. The sections were washed for 10 min in 100 mM Tris-HCl buffer, 150 mM NaCl (pH 7.5), incubated for 30 min in the same buffer (buffer 1) containing 0.5% blocking reagent and 0.2% Triton X-100, and then incubated overnight at room temperature in alkaline phosphatase-conjugated sheep antibodies to digoxigenin diluted to 1/2,000 in buffer 1 or horseradish peroxidase-conjugated sheep antibodies to fluorescein diluted to 1/100 in buffer 1. On the next day, sections were incubated in the NBT/BCIP (nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt) chromogen solution (NBT/BCIP stock solution, Roche) or for fluorescent detection in HNPP (2-hydroxy-3-naphtoic acid-2'-phenylanilide phosphate)/FastRED solution (Roche) for 2–4 h. Finally, slides were coverslipped with Vechashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). They were observed under an epifluorescence microscope (Olympus Provis) equipped with a DP71 digital camera. Images were processed with the Olympus Analysis Cell software. Plates were assembled using Photoshop CS4.

Production antibodies to zebrafish Kiss1 and Kiss2

Chemicals and reagents

L-amino acid residues were purchased from Senn Chemicals (Dielsdorf, Switzerland). The preloaded 4-hydroxymethyl-phenoxymethyl-copolystyrene-1%-divinylbenzene (HMP) resin, O-benzotriazol-1-yl-N, N, N′, N′-tetramethyluronium hexafluorophosphate (HBTU), and 1-hydroxy-benzotriazole (HOBt) were from Applera-France (Courtaboeuf, France). Acetonitrile and N-methylpyrrolidone (NMP) were from Carlo Erba (Val-de-Reuil, France). Diisopropylethylamine (DIEA), piperidine, trifluoroacetic acid (TFA), and other reagents from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

To ensure the specificity of each antisera and avoid potential cross reactions, antibodies were prepared against the preproKiss1 and preproKiss2 C-terminal regions (Supplemental Fig. 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org/). The zebrafish preproKiss1 (102–116) sequence, with an additional Cys^0 residue (CEQDMLTRLKQKSPVK), and the zebrafish preproKiss2 (121–125) sequence, with an additional Cys^0 residue (CQLETS), were synthesized as previously described (28–30). The synthetic peptides were purified by reversed-phase HPLC on a 2.2 × 25-cm Vydac 218TP1022 C_{18} column (Grace Alltech, Templemars, France) using a linear gradient (10–40% over 45 min for CEQDMLTRLKQKSPVK and 2–20% over 40 min for CQLETS) of acetonitrile/TFA (99.9 : 0.1; vol/vol) at a flow rate of 10 ml/min. Peptides were analyzed by reversed-phase HPLC on a Vydac 218TP54 C_{18} column (0.46 × 25-cm; Grace Alltech) using a linear gradient (10–40% over 40 min for CEQDMLTRLKQKSPVK and 2–20% over 40 min for CQLETS) of acetonitrile/TFA at a flow rate of 1 ml/min. The purity of both peptides was higher than 99.9%. The identity of the synthetic peptides was confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Voyager DE-PRO, Applera France).

Immunization protocol

Polyclonal antibodies against the zebrafish preproKiss1 (102–116) and prepro-metastin-2 (121–125) sequences were raised in rabbits. The immunogens were prepared by conjugating CEQDMLTRLKQKSPVK and CQLETS (4 mg each) to 5.5 mg and 15.3 mg maleimide-activated keyhole limpet hemocyanin (MilleGen, Labège, France). For each immunogen, two New-Zealand rabbits (Charles River, L'arbresle, France) were injected intradermally at multiple sites with an equivalent of 200 μg of hapten (reconstituted in 1 ml PBS) emulsified with 1 ml complete Freund's adjuvant for the first injection and 1 ml incomplete Freund's adjuvant for subsequent injections (Sigma-Aldrich). Injections were repeated at monthly intervals, and blood samples were collected 1 week after the third and subsequent immunizations.

Immunohistochemical detection of zebrafish preproKiss1 and preproKiss2

To verify the specificity and quality of the developed Kiss antibodies, cryostat sections were rinsed in PBS and incubated for 1 h at 80 C in Tris HCl 50 mM (pH 8) before nonspecific binding was blocked in 0.2% Triton PBS containing 0.5% blocking reagent at room temperature. Sections were then incubated overnight at room temperature with primary antibodies of interest in PBS containing 0.5% blocking reagent. Sections were washed three times in 0.2% Triton PBS and incubated with antirabbit and anti-guinea pig Alexa fluor 594 or 488 (1:200; Invitrogen Molecular Probes, Eugene, OR) for 1.5 h at room temperature. After washing in PBS, slides were coverslipped with Vectashield mounting medium with DAPI (Vector Laboratories). The slides were observed with an epifluorescence microscope (Olympus Provis), and images were processed with the Olympus Analysis Cell software. The nomenclature is according to Wullimann *et al.* (54).

Double immunohistochemical detection of zebrafish preproKiss2 and preproGnRH3

To study the potential interactions between GnRH3 and Kiss systems, double immunochemical techniques were performed. The GnRH3 antibody used in this study has been produced in guinea pig against the GnRH-associated peptides (GAP) fragment of sea bass preproGnRH3 (31) that shows 80% of identity with the GAP fragment of zebrafish GnRH3. The specificity of this antiserum was verified by double staining with a salmon GnRH antibody (31, 32), which cross-reacts with GnRH2. Double staining showed perfect coexpression of the two signals in the anterior brain GnRH3 systems, while the GnRH2 systems were not labeled by the sea bass preproGnRH3 antibody (data not shown).

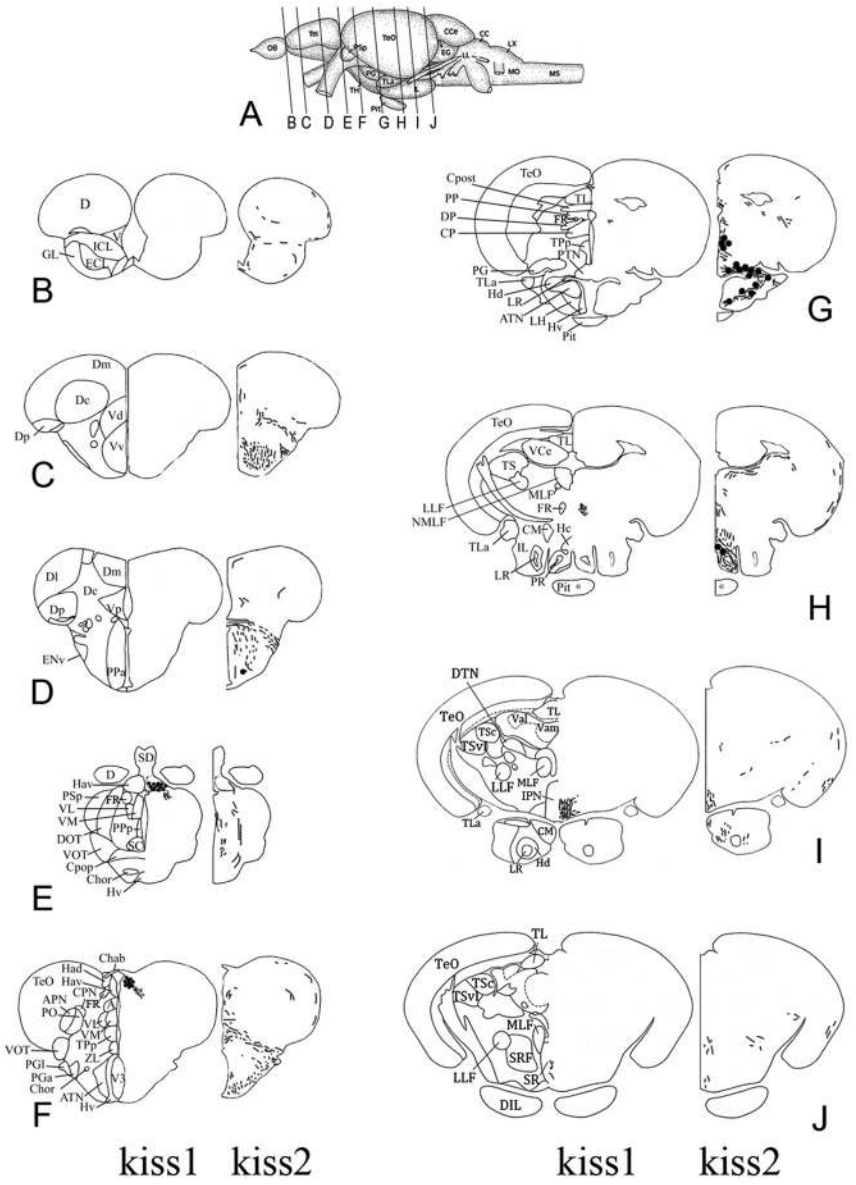

FIG. 1. Schematic representation of *kiss1* and *kiss2* expressing cells on representative transverse sections (B–J), taken from the zebrafish brain atlas (54). The level of these sections is shown in A, taken from the atlas. *Black dots* indicate the location of cells expressing *kiss1* (*left*) or *kiss2* (*right*). *Dotted lines* represent the distribution of the ir fibers. A, Anterior thalamic nucleus; APN, accessory pretectal nucleus; ATN, anterior tuberal nucleus; CC, crista cerebellaris; CCe, corpus cerebelli; Chab, habenular commissure; Chor, horizontal commissure; CM, corpus mamillare; CP, central posterior thalamic nucleus; CPN, central pretectal nucleus; Cpop, postoptic commissure; Cpost, posterior commissure; D, dorsal telencephalic area; Dc, central zone of dorsal telencephalic area; Dl, lateral zone of dorsal telencephalic area; DIL, diffuse nucleus of the inferior lobe; Dm, medial zone of dorsal telencephalic area; DOT, dorsomedial optic tract; Dp, posterior zone of dorsal telencephalic area; DP, dorsal posterior thalamic nucleus; DTN, dorsal tegmental nucleus; ECL, external cellular layer of olfactory bulb; EG, eminentia granularis; ENv, entopendoncular nucleus, ventral part; FR, fasciculus retroflexus; GL, glomerular layer of olfactory bulb; Had, dorsal habenular nucleus; Hav, ventral habenular nucleus; Hc, caudal zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; Hv, ventral zone of periventricular hypothalamus; ICL, internal cellular layer of olfactory bulb; IL, inferior lobe; IPN, interpeduncular nucleus; LH, lateral hypothalamic nucleus; LL, lateral line nerves; LLF, lateral longitudinal fascicle; LR, lateral recess of diencephalic nucleus; LX, lobus vagus; MLF, medial longitudinal fascicle; MO, medulla oblongata; MS, medulla spinal; NMLF, nucleus of medial longitudinal fascicle; OB, olfactory bulb; PG, preglomerular nucleus; PGa, anterior preglomerular nucleus; PGl, lateral preglomerular nucleus; Pit, pituitary; PP, periventricular pretectal nucleus; PO, posterior pretectal nucleus; PPa, parvocellular preoptic nucleus, anterior part; PPp, parvocellular preoptic nucleus, posterior part; PR, posterior recess of diencephalic ventricle; PSp, parvocellular superficial pretectal nucleus; PTN, posterior tuberal nucleus; SC, suprachiasmatic nucleus; SD, saccus dorsalis; SR, superior raphe; SRF, superior reticular formation; Tel, telencephalon; TeO, tectum opticum; TH, tuberal hypothalamus; TL, torus longitudinalis; TLa, torus lateralis; TPp, periventricular nucleus of posterior tuberculum; TS, torus semicircularis; Tsc, central nucleus of semicircular torus; TSv1, ventrolateral nucleus of semicircular torus; V, ventral telencephalic area; V3, third ventricle; Val, valvula cerebella pars lateralis; Vam, valvula cerebella pars medialis; VCe, valvula cerebelli; Vd, dorsal nucleus of ventral telencephalic area; VL, ventrolateral thalamic nucleus; VM, ventromedial thalamic nucleus; VOT, ventrolateral optic tract; Vp, postcommissural nucleus of ventral telencephalic area; Vv, ventral nucleus of dorsal telencephalic area; ZL, zona limitans.

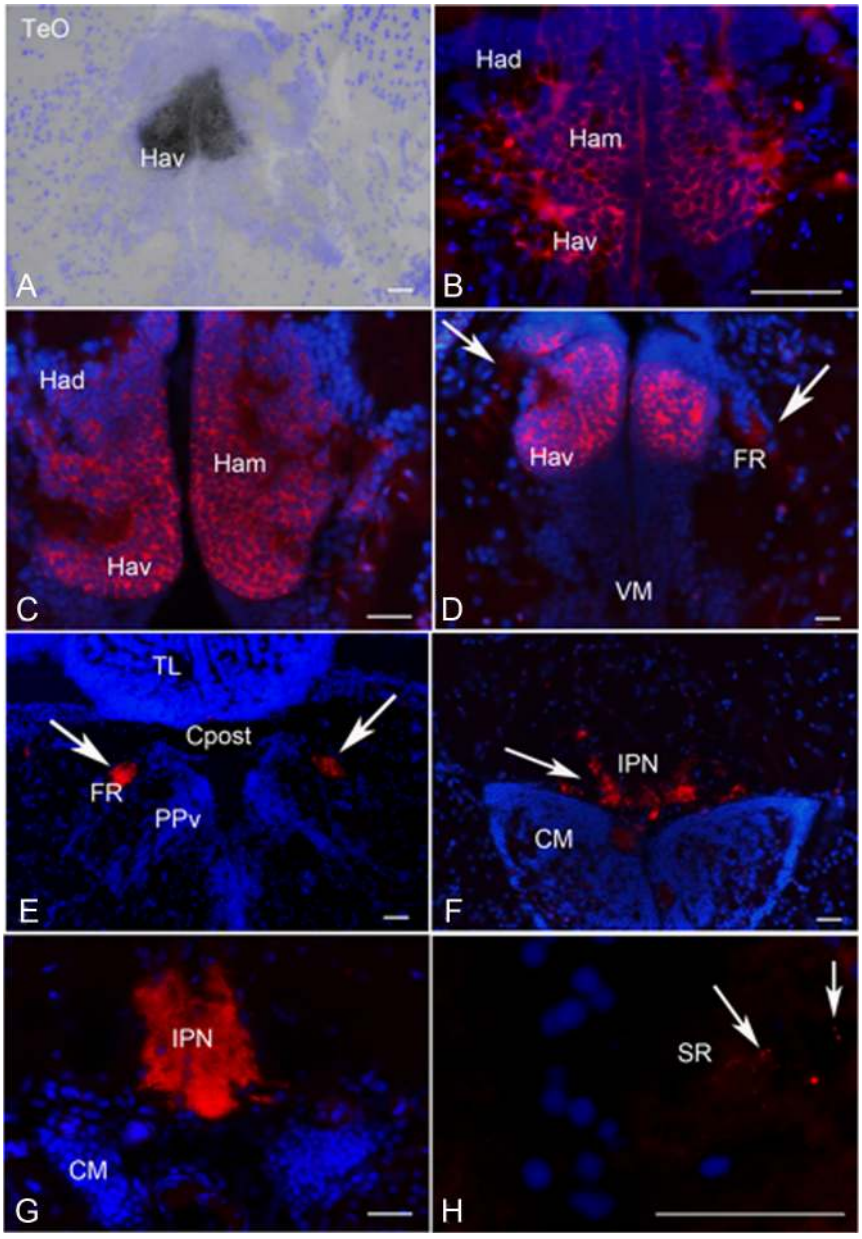

FIG. 2. Localization of *kiss1*-expressing cells as indicated by *in situ* hybridization (A and B) and immunohistochemistry (C–H). *Kiss1* mRNA-expressing cells are consistently observed in the ventral (Hav) and medial (Ham) but not dorsal habenula (Had) whatever the technique used, NBT/BCIP (A) or HNPP/FastRED (B). Immunohistochemical labeling using the preproKiss1 antibody also caused staining of cells in the same habenular regions (C and D) and their projections through the fasciculus retroflexus (FR, *arrows* in D and E) down to the interpeduncular nucleus (IPN). These fibers entered the IPN through its ventrolateral margin (F, *arrow*) and more caudally stained the entire extent of the ventral interpeduncular nucleus (G). In H, scattered fibers were also observed in the superior raphe nucleus (SR, *arrows*). Adjacent sections incubated with the sense probe showed no signal. *Scale bar*, 20 μm.

Zebrafish exposure to estradiol and RNA extraction

Juvenile zebrafish 2 months of age were exposed to 10^{-8} M estradiol (β-estradiol, Sigma-Aldrich, St. Louis, MO) or vehicle for 7 d in glass tanks. The water was maintained at 28.5 C and replaced every day. After exposure, pools of five brains were sonicated (10 sec, three times) in 1 ml Trizol Reagent (Life Technologies, Inc., Carlsbad, CA), and total RNA was extracted according to the manufacturer's protocol.

Quantitative real-time PCR

Reverse transcription was carried out by incubating 2 μg total RNA with 5 mM random examer oligonucleotides, 10 mM dithiothreitol, 2.5 mM deoxynucleotide triphosphate, and 100 U Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) in the appropriate buffer for 30 min at 37 C and 15 min at 42 C. PCR reactions were performed in an iCycler hermocycler coupled to the MyiQ detector (Bio-Rad, Hercules, CA) using iQ SYBR-Green Supermix (Bio-Rad) according to the manufacturer's protocol. The following primers were used: elongation factor 1 (EF-1) (fw) 5′-AGCAGCAGCTGAGGAGTGAT- 3′, EF-1 (rev) 5′-CCGCATTTGTAGATCAGATGG-3′; Kiss1 (fw) 5′-CAAGCTCCATACCTGCAAGTG-3′, Kiss1 (rev) 5′-GTACCCTCGCCACTGACAAC-3′; Kiss2 (fw) 5′- AGGAGGACAGCAGAGAATGG-3′, Kiss2 (rev) 5′- GCGCCCTGGTATTCATATTG-3′; Kiss1r (fw) 5′- TCAACAGGTGACGGTACAGG-3′, Kiss1r (rev) 5′- ATGGTGCAGGGATTTGAGAG-3′; Kiss2r (fw) 5′- CCGTTCAAGGCTGTAACCAG-3′, Kiss2r (rev) 5′- CAGCACCATCACCACTACCA-3′. Expression levels of EF-1 mRNA were used to normalize the expression levels of the other genes. Melting curve and PCR efficiency analyses were performed to confirm a correct amplification. Each experiment was performed at least twice in triplicate and significant of fold induction was determined with a Student's *t* test.

Control procedures

All plasmids have been sequenced to check correspondence with the published sequence information. In the case of *in situ* hybridization, parallel sections were always hybridized with the sense probe, yielding negative results (Supplemental Fig. 2). Dot blots showed that the antibodies indeed recognize the corresponding peptide and do not show any cross-reactivity even at low antibody dilution (data not shown). In the case of immunohistochemistry, adjacent sections were exposed to antibodies to Kiss1 and Kiss2 preincubated with CEQDMLTRLKQKSPVK and CQLETS, respectively.

Results

Localization of *kiss1* and *kiss2* mRNA-expressing cells in adult zebrafish brain

In situ hybridization techniques used clearly showed the segregation of *kiss1*- and *kiss2*-expressing cells in anatomically distinct areas of the adult zebrafish brain with

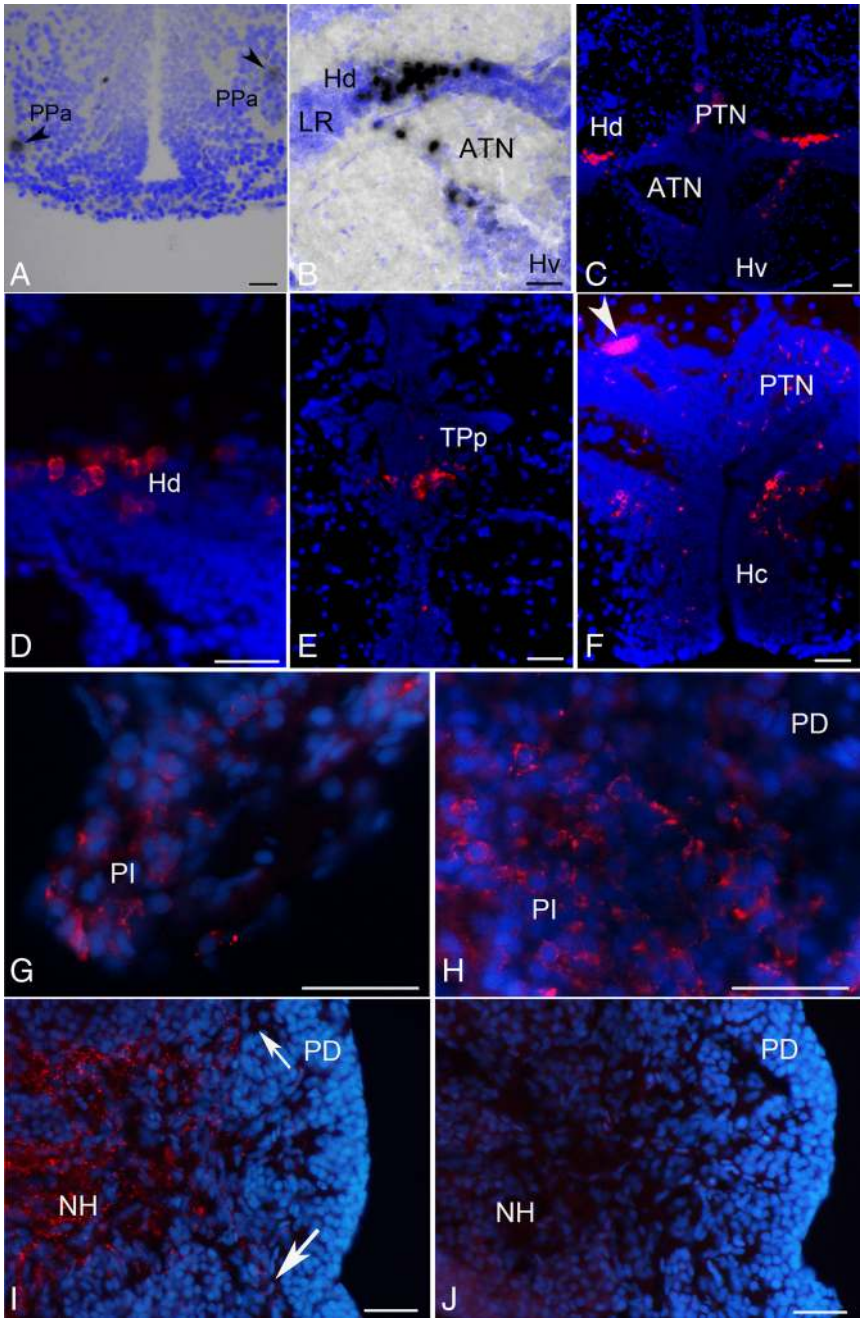

FIG. 3. A, Few Kiss2-expressing cells were detected in the anterior parvocellular preoptic nucleus (PPa, *black arrowheads*). B–F, *In situ* hybridization of *kiss2* mRNA-expressing cells in the hypothalamus, at the level of the dorsal hypothalamus (Hd). These cells were observed dorsal to the lateral recess (LR) in its anterior part (A–D) and also surrounding the anterior tuberal nucleus (ATN). In E and F, cells were also detected in the periventricular nucleus of the posterior tuberculum (TPp) and caudal hypothalamus (Hc). Identical results were obtained with the NBT/BCIP (A and B) or HNPP/FastRED (C and D) revelation methods. G and H, Immunohistochemical labeling using the preproKiss2 antibody showed the presence of many ir cells in the pars intermedia (PI) of the pituitary, whereas none could be detected in the pars distalis (PD). I and J, Immunohistochemical labeling using anti-GnRH3 (I) or preproKiss2 (J) antibodies showed the presence of a dense GnRH3 innervation within the neurohypophysis (NH), invading the pars distalis (*arrows*), while, on the adjacent section (J), absolutely no proKiss2-ir fibers could be detected. In F, *arrowhead* points to a large blood vessel. *Scale bar*, 20 μm.

no obvious sexual differences (Fig. 1). *Kiss1* RNA-containing cells were exclusively observed in the ventromedial habenula (Figs. 2, A and B, and 8A). This kiss1 neural population consists of many densely packed small cells exhibiting a round nucleus and a thin ring of cytoplasm. *Kiss2*-expressing cells were consistently detected in the mediobasal hypothalamus (Fig. 1) mainly in proximity of the lateral recess surrounding its superior extent or along the lateral wings of the ventromedial hypothalamus (Figs. 3, B–F, and 8, C–E). A small number of Kiss2 neurons were detected into the preoptic area in a number of specimens (Figs. 1D and 3A). Additionally, weakly stained neurons were detected in the ventral hypothalamus in some individuals (Figs. 1G and 8E). A group of *kiss2* mRNA-positive cells were also detected in the posterior tuberal nucleus (Fig. 1G) in a more medial position. Finally, another kiss2-expressing cell population was observed at the level of the periventricular nucleus of the posterior tuberculum (Figs. 1G and 3E).

Immunohistochemical study of the kiss1 and kiss2 systems in zebrafish brain and pituitary

Further confirming the specificity of the antibody, preproKiss1-immunoreactive (ir) neurons were strictly localized into the ventromedial habenula. (Figs. 1, E and F, and 2, C and D). Moreover, proKiss2 cells were consistently observed in the mediobasal hypothalamus, mostly spread along the upper border of the lateral recessus and the lateral wings of the ventral hypothalamus (Figs. 1 and 4, E and F). Positive cells were also seen in the periventricular nucleus of the posterior tuberculum. No other populations of proKiss1- and proKiss2-expressing cells were detected by immunohistochemistry, except in the pituitary with the proKiss2-antibody.

The projections of proKiss1-positive neurons further confirmed the specificity of the antibody. Indeed, as expected, proKiss1 neurons of the ventromedial habenula sent axons caudally forming the fasciculus retroflexus (Figs. 1, E–I, and 2, D–F). This fascicle that courses laterally from the habenulae was intensely immunostained along its entire extent (Fig. 2, D and E). It could be traced

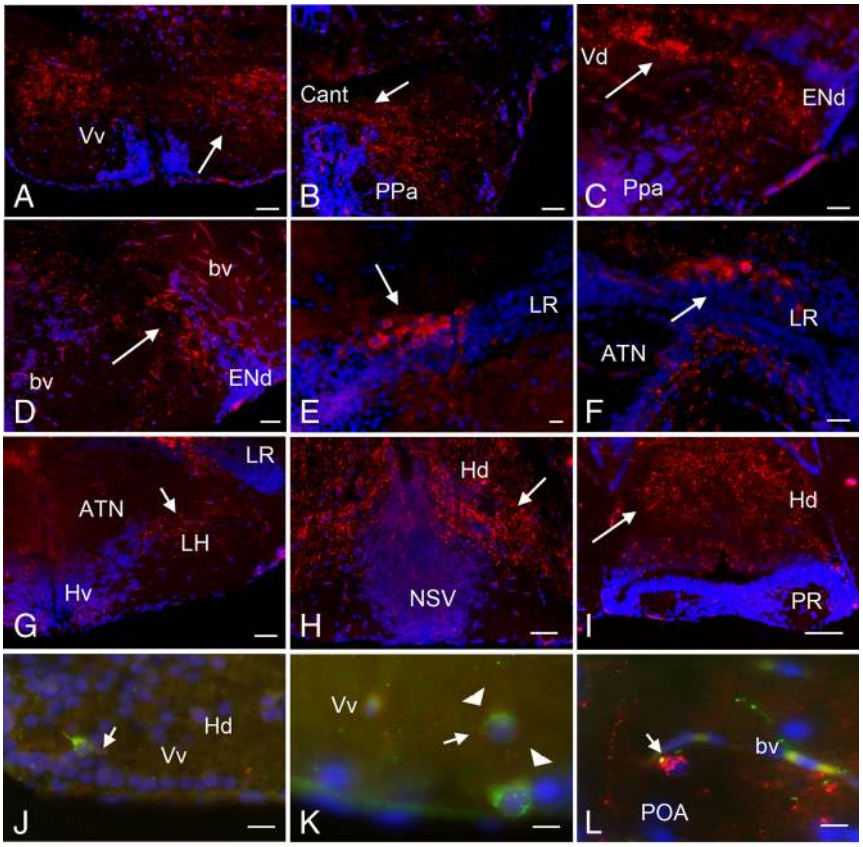

FIG. 4. Immunohistochemistry with preproKiss2 antibodies indicated the existence of a prominent innervation of (A) the subpallium [ventral telencephalon (Vv); *arrow*], (B) the anterior parvocellular preoptic nucleus (PPa) and anterior commissure (Cant, *arrow*). In C and D, a conspicuous projection (*arrow*) was observed to the dorsal entopeduncular nucleus (End), lateral to the dorsal subpallium (Vd). E and F, PreproKiss2-ir fibers leave the cells located dorsal to the lateral recess (LR) and go around the recess, before arching toward the mediobasal hypothalamus. G–I, ProKiss2-ir cells located in the dorsal hypothalamus (Hd) above the lateral recess (LR) send prominent projections around the anterior tuberal nucleus (ATN) toward the ventromedial (Hv) and caudal hypothalamus, notably above the nucleus of the saccus dorsalis (NSV) and above the posterior recess (PR). J–L, Double stainings with the rabbit preproKiss2 antiserum (*green* in J and K, *red* in L) and the guinea pig preproGnRH3 antiserum (*red* in J and K, *green* in L), showing that GnRH3 neurons are contacted by proKiss2 fibers. bv, blood vessels. *Scale bar*, 20 μm (A–I); 10 μm (J); 5 μm (K); 8 μm (L).

down to the ventral tegmentum in the interpeduncular nucleus (IPN) where a dense network of immunopositive proKiss1-ir fibers was observed entering the ventrolateral margin of the IPN (Fig. 2F) and strongly innervating the whole ventral extent of this nucleus (Fig. 2G). In contrast, the dorsal IPN did not exhibit staining. Lastly, weakly stained proKiss1-ir fibers were found into the raphe nucleus (Fig. 2H). No proKiss1-positive fibers could be observed in the pituitary.

Similarly, no proKiss2-ir fibers were detected in the pituitary (Fig. 3, G, H, and J). In contrast, moderately stained cells were obvious in the pars intermedia of the pituitary (Fig. 3, G and H) but not in the pars distalis (Fig. 3H). Figure 3, I and J, shows that, while many GnRH3-ir fibers were present in the neurohypophysis (Fig. 3I), no fibers reacted with the preproKiss2 antiserum.

Within the brain, proKiss2-ir fibers formed a wide network of projections mostly spread into the anterior and midbrain (Figs. 1 and 4). Few proKiss2-ir fibers were revealed into the posterior parts of the olfactory bulbs (Fig. 1B). More caudally, in the telencephalon, the subpallial (Figs. 1C and 4A) and, to a lower extent, the pallial regions (Fig. 1, C and D) exhibited proKiss2-ir fibers. The anterior part of the parvocellular preoptic nucleus also contained an intense proKiss2 innervation made up of thin fibers mainly in the lateral preoptic area before crossing the anterior commissure (Figs. 1D and 4B). Prominent proKiss2 projections were observed running toward, and within, the entopeduncular nucleus including both the dorsal (Figs. 1C and 4, C and D) and ventral portions (Fig. 1D). In more posterior diencephalic regions, proKiss2-ir fibers were revealed in the dorsal and ventral thalamus (Fig. 1, E–G), and a few projections entered the habenulae. A conspicuous bundle of proKiss2 projections was found leaving the periventricular nucleus of the posterior tuberculum. These fibers extended laterally and arched ventrally at the level of the preglomerural nuclei before descending in the caudal hypothalamus (Figs. 1, F and G, and 4, E–I). At the level of the hypothalamus, a very obvious innervation was observed surrounding the lateral recess in the regions where most immunostained proKiss2 perikarya were observed (Fig. 1, G and H). Projections were also labeled at the outer margin of the lateral wings of the ventral hypothalamus (Fig. 4, F and G). Such fibers then entered the ventral hypothalamus and remained very abundant up to the most caudal portion of the hypothalamus (Fig. 4, H and I). From the caudal hypothalamus, proKiss2-immunopositive fibers were identified ascending dorsally into some mesencephalic nuclei including the torus semicircularis and the nucleus of the medial longitudinal fascicle (Fig. 1, H–J). Finally, some proKiss2-ir fibers were observed in the optic tectum (Fig. 1, F–J). No significant innervation was detected in more caudal brain regions.

Double immunohistochemical detection of zebrafish proKiss2 and proGnRH3

Double stainings were performed with the proGnRH3 antiserum raised in guinea pigs, revealing scattered

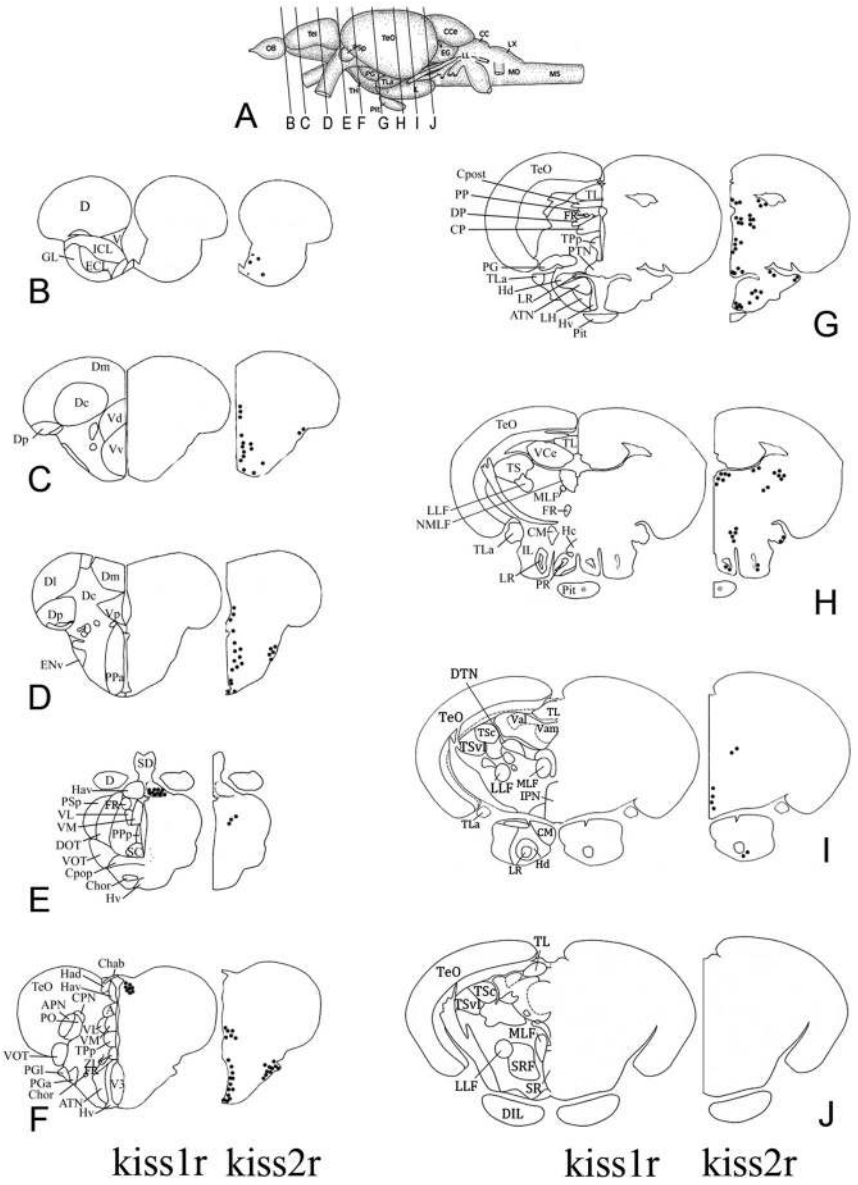

FIG. 5. Schematic representation of *kiss1r*- and *kiss2r*-expressing cells on representative transverse sections (B–J), reproduced with permission from Wullimann *et al.* (54). The level of these sections is shown in A, reproduced with permission from Wullimann *et al.* (54). *Black dots* indicate the location of cells expressing *kiss1r* (*left*) or *kiss2r* (*right*). Abbreviations as in the legend to Fig. 1.

GnRH3 neurons in the subpallium and the anterior preoptic area with fibers running rostro-caudally toward the pituitary stalk. Some of these preoptic GnRH neurons were contacted by proKiss2 fibers as shown on Fig. 4, J–L.

Neuroanatomical localization of kiss1r and kiss2r-expressing cells in the brain of adult zebrafish

The only site of expression of *kiss1r* mRNA cells was the ventromedial habenula, showing an expression pattern very similar to that of the *kiss1* mRNA or proKiss1 peptide (Figs. 5, E and F, and 6, A–D). Double staining indicated that cells expressing *kiss1* were the same as those expressing the *kiss1r* messengers (Fig. 6, E–H).

In contrast, the sites of *kiss2r* mRNA expression were more widespread. In the anterior brain, very few *kiss2r*-expressing cells were observed in the caudal region of the olfactory bulb, while their density was higher in the ventral and dorsal portions of the subpallium (Figs. 5, B and C, and 7A). A high density of *kiss2r*-messengers was observed in the parvocellular preoptic nuclei, notably its anterior part (Figs. 5D and 7, C and D), as well as in the entopeduncular nucleus (Figs. 5, C and D, and 7B). A moderate expression of *kiss2r* messengers was observed in the thalamus and pretectal regions (Figs. 5, F and G, and 7, E and F). More posteriorly, regions with *kiss2r*-mRNA expression were the posterior tuberculum, the ventral hypothalamus, and the posterior tuberal nucleus (Figs. 5, F and G, and 7, E–G). In a more lateral position *kiss2r*-expressing cells appeared near and within the preglomerular nuclei (Fig. 5F). Finally, the IPN and the torus semicircularis also expressed *kiss2r* (Fig. 5, I and J). Expression of *kiss1r* and *kiss2r* was not examined in the pituitary.

Estradiol affects Kiss expression in juvenile zebrafish

To see whether the Kiss systems are sensitive to estrogens, juvenile zebrafish were exposed to 10^{-8} M estradiol for 7 d, resulting in a significant increase in the number on *kiss2* neurons detectable by *in situ* hybridization in the hypothalamus. This increase was most obvious at the periphery of the anterior tuberal nucleus (Fig. 8, A and B) and in the caudal hypothalamus (Fig. 8, C and D). These data were confirmed by the fact that E2 causes a significant increase of *kiss2* mRNAs as measured by RT-PCR. *Kiss1* mRNAs were also significantly elevated, but to a much lesser extent. Finally, as assessed by RT-PCR, *kiss2r* expression was increased, but not that of *kiss1r* (Fig. 8E).

Discussion

To our knowledge, this study is the first to document in detail the organization of two independent kiss systems in the brain of a teleost fish, the distribution of the corre-

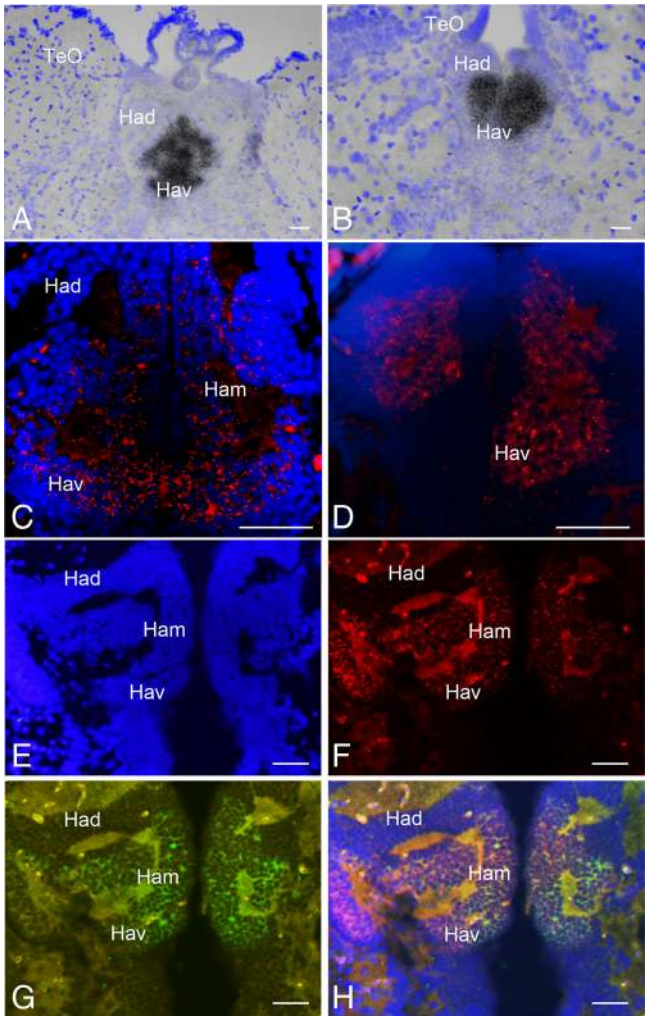

FIG. 6. A–D, *In situ* hybridization of *kiss1r* using two different revelation methods, NBT/BCIP (A and B) or HNPP/FastRED (C and D), both showing strong expression restricted to the ventral (Hav) and medial (Ham) extents of the habenulae, while the dorsal part (Had) was not labeled. E–H, Transverse section of the habenular region stained with DAPI (E), hybridized with the *kiss1r*-probe (F), before being immunostained with the preproKiss1 antiserum (G). The merge in H shows the virtually perfect overlap between the two signals in the ventral habenula while the dorsal component was not labeled. *Scale bar*, 20 μm.

sponding receptors, and the potential sites of interactions with the GnRH systems and estrogen signaling (Fig. 8). Importantly, this study also presents an interesting strategy to raise specific antibodies against prokisspeptins.

PreproKiss1 and preproKiss2 antibodies

The study of the Kiss systems in vertebrates has been, and still is, impaired by the difficulty encountered to raise specific antibodies to kisspeptins. Indeed, cross reactivity with peptides of the RFamide family is often observed (14). Using antibodies to human KISS1 (9), we could detect fish Kiss1 (YNLNSFGLRY-NH_2) and Kiss2 (FNYNPFGLRF-NH_2) decapeptides, given their sequence similarity with mammalian KISS. However, these antibodies were not usable, first because they obviously showed some cross-reactivity with other fish peptidergic systems, and second because they were unable to discriminate between zebrafish Kiss1 and Kiss2 as evidenced by dot blot (data not shown). To circumvent these problems, we decided to generate antibodies against the C-terminal regions to avoid potential cleavage sites existing in other regions of the precursors. Such a strategy was already successfully used to generate specific antibodies to three GnRH variants existing in the sea bass, a teleost fish. In that case, antibodies to the GAP proved very efficient to study the organization of the three GnRH systems (31, 33, 34). The strategy developed in this study was also successful to raise antibodies specific for the two Kiss-precursors. Dot blots indicated very good specificity of these antibodies and the lack of cross-reactivity. Furthermore, the excellent correspondence between localization of *kiss1* or *kiss2* mRNAs and proteins also reinforces the conclusion that the antibodies developed are indeed suitable for the study of the Kiss1 and Kiss2 systems in zebrafish. The fact that extensive projections could be traced from the proKiss1 and proKiss2 neurons indicates that, as it was already shown GnRH (35), accompanying peptides travel in the axons in a way similar to the active peptide.

Kiss1 and Kiss1r are expressed in the habenula

The only site where we could detect *kiss1 mRNA* is the ventromedial habenular nucleus. This is similar to a previous report in zebrafish and medaka (20). In medaka, however, *kiss1* is also weakly expressed in the hypothalamus (20). The finding of kiss1 mRNAs in the habenula is now substantiated by the demonstration of the proKiss1 protein in that same structure. In fish, the habenular complex is divided into two separate regions, the ventral habenula and the dorsal habenula, both having complex tridimensional asymmetrical patterns. According to recent data in zebrafish, the ventral habenula is homologous to the mammalian lateral habenula and projects to the raphe nucleus. In contrast, the dorsal habenula would be homologous to the mammalian lateral habenula and, as such, massively projects to the IPN via the fasciculus retroflexus (36, 37). The fasciculus retroflexus is a prominent fiber tract that carries efferent axons from the habenula toward the targets in the midbrain/hindbrain, notably to the IPN. This pathway is strongly conserved in vertebrates and is part of the so-called dorsal diencephalic conduction system that links the forebrain to the mid- and hindbrain (38). Accordingly, prominent Kiss1 staining was observed in the fasciculus retroflexus and could be traced down to the IPN. In addition, scattered Kiss1-ir fibers were seen in the raphe region. Our results and those obtained previously (20) show that *kiss1* is expressed in the ventral

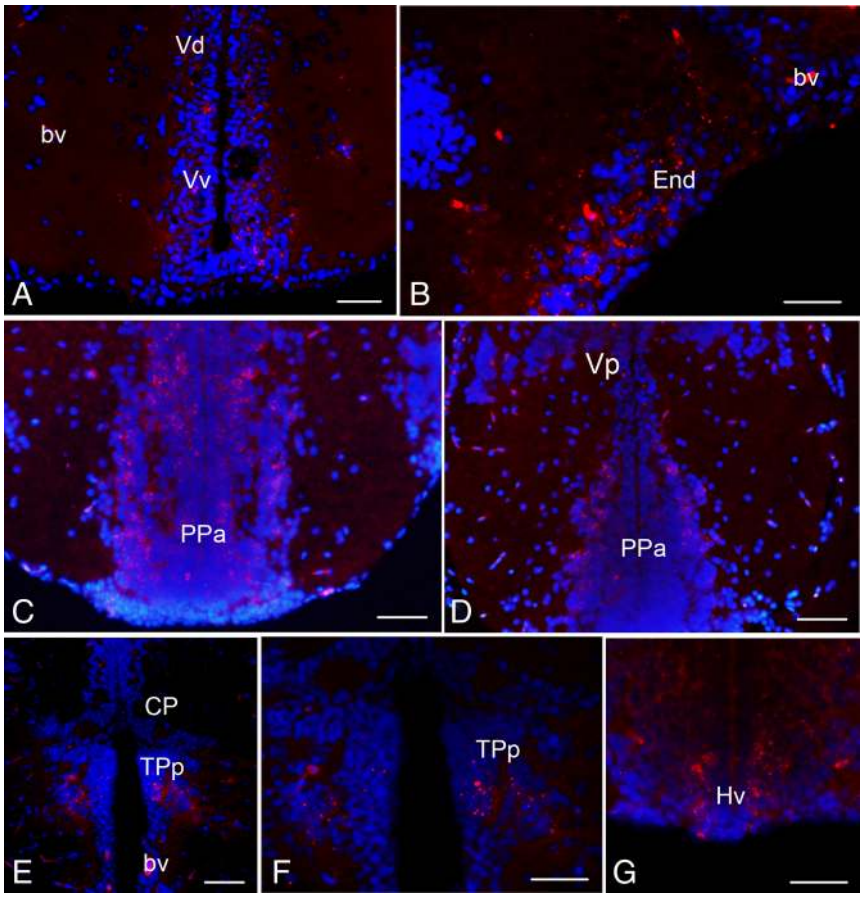

FIG. 7. *In situ* hybridization of *kiss2r* messengers in various forebrain regions: (A) dorsal (Vd) and ventral (Vv) subpallium, (B) dorsal entopeduncular nucleus (End), (C and D) anterior parvocellular preoptic nucleus (PPa), (E and F) periventricular nucleus of the posterior tuberculum (TPp), and (G) mediobasal hypothalamus (Hv). *Scale bar*, 20 μm.

habenula and part of the medial habenula, which is consistent with the fact that we observed projections in both the IPN and the raphe region. Apart from the previous report in the same species (20), no one has ever mentioned KISS expression in the habenulae, notably in mammals where KISS1 expression is now well documented (9, 39, 40).

Very surprisingly, the present study revealed strong expression of kiss1r on kiss1-expressing neurons. Using combinations of proKiss1 immunohistochemistry and *kiss1r in situ* hybridization, we could demonstrate the perfect overlap between the two signals, indicating that these neurons provide an interesting case of autocrine regulation. No other sites of expression of *kiss1r* could be detected. Although *kiss2*r expression was observed in the IPN, this expression was not massive in view of the very heavy innervation of the ventral portion of the IPN by Kiss1 fibers. Recent biochemical data indicated that pyroGlu-kisspeptin-15 (pENVAYYNLNSFGLRY-NH_2), derived from zebrafish preproKiss1, has very high affinity for the kiss1r (16). It is thus likely that Kiss1 neurons are autoregulated by their own product.

The massive innervation of the IPN by Kiss-1 fibers is in line with the well-known projections of habenular neurons in this structure in fish (36, 41) and other vertebrates (38). The IPN is considered an important integrative center and relay location within the limbic system in mammals (42). In mammals, it is involved in a variety of functions, such as maternal and reproductive behavior, aversive responses, circadian rhythms, sleep, or control of motor activities through the dopaminergic systems (43). The projections of Kiss1 neurons only in the IPN suggests that the Kiss1 system neither plays a role in the control of pituitary functions nor in the control of GnRH neurons in fish.

The hypothalamic Kiss2 neurons: the link to GnRH via the kiss2r receptor

Several populations of cells expressed Kiss2 in the zebrafish hypothalamus: the lateral preoptic area, the posterior periventricular nucleus, nucleus of the lateral recess, nucleus lateralis tuberis, and its lateral wings. This further details information on kiss expression obtained in the zebrafish (20) or the medaka (19) based on *in situ* hybridization only. We now report for the first time in teleost fish accurate data on the projections of these neurons in many forebrain and midbrain locations. A remarkable feature of all these projections is the fact that they match virtually perfectly the distribution of *kiss2r*-expressing cells, further reinforcing the validity of our results. Similar to the mouse (15), *kiss2r* messengers have wide distribution, notably in the subpallium, preoptic area, thalamus and hypothalamus, IPN, and midbrain tegmentum. It is thus very likely that Kiss2 neurons target cells expressing *kiss2r*. Among potential targets of Kiss2 are the GnRH neurons that are located along a continuum extending from the olfactory bulbs to the anterior hypothalamus (32, 44–46). The present work shows that regions exhibiting GnRH3 neurons receive proKiss2 fibers with kiss2 punctates occasionally observed on GnRH3 perikarya. Until now, we did not succeed in coupling *kiss2r* expression with GnRH3 immunohistochemistry, but in another teleost, the European sea bass, *kiss2r* were detected in GnRH1 neurons, homologous to GnRH3 of zebrafish (S. Escobar and A. Servili,

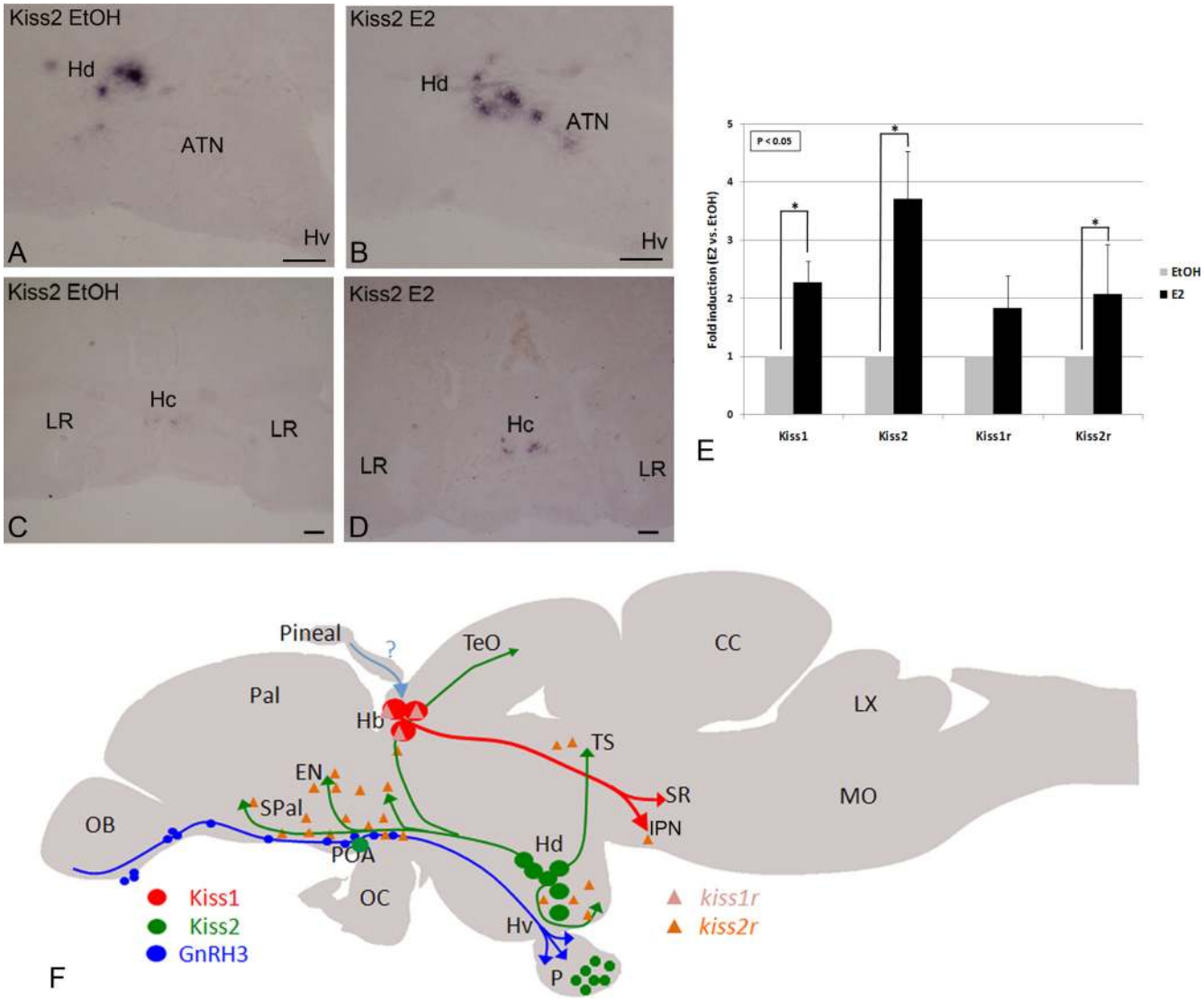

FIG. 8. A–D, *In situ* hybridization of *kiss2* in control (A and C) and E2-treated fish (B and D) showing the increase in the number of neurons expressing kiss2 in the dorsal (Hd) or caudal (Hc) hypothalamus and at the periphery of the anterior tuberal nucleus (ATN).: *Scale bar*, 20 μm (A and B); 40 μm (C and D). E, Estradiol (10^{-8} M) treatment causes a significant increase in both kiss1 and kiss2 mRNA expression in the brain of 2-month-old juvenile zebrafish. The effect is more pronounced on *kiss2* mRNAs. E2 also causes an increase in *kiss2r* receptors messengers. F, Schematic representation of the organization of the Kiss1 and Kiss2 systems in the brain of zebrafish. Kiss1 neurons are restricted to the habenula (Hb). They express also kiss1r and project only into the IPN and the superior raphe (SR). Kiss2 neurons are located in the dorsal (Hd), lateral, and ventral hypothalamus (Hv). These neurons send extensive projections toward the subpallium, the entopeduncular nucleus (EN), the preoptic region (POA), the thalamus, the ventral and caudal hypothalamus, and the torus semicircularis (TS). In all these regions, *kiss2r* are widely expressed. Kiss2 fibers make direct contacts with GnRH3 neurons, which project to the pituitary. In contrast, no Kiss fibers were seen in the pituitary (P), whereas Kiss2-positive cells are present in the pars intermedia. CC, Crista cerebelli; LX, vagal lobe; MO, medulla oblongata; OB, olfactory bulb; OC, optic chiasma; Pal, palium; TeO, optic tectum.

unpublished data). Thus, as already suggested in tilapia (25), GnRH neurons are likely to be a target of Kiss2 acting through kiss2r receptors. In agreement, zebrafish *kiss2r* have more affinity than *kiss1r* for Kiss2 ligand (16). This is also consistent with the fact that Kiss2 is more efficient than Kiss1 in regulating the synthesis or release of gonadotrophins (17, 20). Furthermore, Kiss2, but not Kiss1 neurons, appeared regulated by estrogens in the zebrafish (47). This set of data strongly suggests that kiss2 neurons in zebrafish are involved in the neuroendocrine control of gonadotrophin release through the GnRH system.

Kiss2 neurons are sensitive to estradiol

While estradiol treatment of juvenile zebrafish caused increased expression of *kiss1* messengers, the effects on *kiss2* were much more pronounced and also visible by *in situ* hybridization because estrogen treatment caused the appearance of cells in the ventral and caudal hypothalamus. Interestingly, the medaka exhibits in the same loca-

tion an estrogen-sensitive population that expresses *kiss1*. The zebrafish *kiss2* neurons of the ventral hypothalamus would then be homologous to the estrogen-sensitive *kiss1* hypothalamic neurons in the medaka (47). Obviously, similar to the three GnRH genes whose sites of expression vary between teleost orders (48), the kiss systems in teleosts will likely show important variations between species.

The pituitary expresses proKiss2, but does not receive kiss fibers

The zebrafish pituitary does not receive any proKiss1 or proKiss2 fibers, which is in agreement with the fact that, with the exception of the ventral hypothalamus, none of the nuclei exhibiting either *kiss1* or *kiss2* expression are known for projecting to the pituitary of teleosts (49). Accordingly, no fibers were observed running toward the pituitary stalk. This is in line with the fact that little KISS fibers are observed in the external zone of the median eminence (14). In contrast, we detected abundant proKiss2-ir cells in the pars intermedia of the pituitary that in fish contains a-MSH and somatolactin-expressing cells (44, 50). At the present time, it is not known whether the zebrafish pituitary expresses *kissr* and where. In the rat, both KiSS-1 and GPR54 where shown to be expressed in LH cells (51). It is also possible that Kiss2 produced by the pars intermedia acts in a paracrine manner onto the gonadotrophs. Such a paracrine regulation has already been proposed in the case of [Pro2, Met13] somatostin (SS2) expressed in the frog melanotrophs and potentially acting on growth hormones cells (52).

Comparative and phylogenetic considerations

As stated above, the number of kiss genes varies from none in birds to three in amphibians, indicating that the kiss systems have markedly changed during evolution. Phylogenetical studies strongly suggest that the Kiss1 and Kiss2 genes were generated by duplication of ancestral chromosome/paralogon (16, 17). In mammals, KISS1 is expressed in both the preoptic area and the ventral hypothalamus (9, 14, 39, 40), and there is no KISS2 gene (16, 17). In *Xenopus*, the preoptic area and the ventromedial hypothalamus express both *kiss1* and *kiss2* mRNAs and the Kiss2 neurons send axons to the median eminence (16). In the very diversified and evolutionary ancient group of teleost fishes, there is growing indication that the organization of these systems are differing between zebrafish (Ostariophysi Cypriniformes), medaka (Acanthopterygii, Beloniformes), sea bass (Acanthopterygii, Perciformes), and fugu (Acanthopterygii, Tetraodontiforms). While zebrafish strongly express *kiss1* in the habenula and *kiss2* in the ventral hypothalamus (20), *kiss1* habenular expression seems to decrease in more evolved fish such as sea bass (S. Escobar and A. Servili, unpublished). In the fugu that has lost the *kiss1* gene (53), expression of Kiss in the habenula is not documented. It thus seems that in tetrapods and fishes there would be a similar evolutive trend toward the emergence of a prominent kisspeptin system expressed in both the preoptic area and ventral hypothalamus (53).

In conclusion, we provide, for the first time in fish, detailed information on the organization of two independent kiss systems in teleosts. We show that the zebrafish *kiss2* gene is likely involved in the control of reproductive functions, notably through interactions with the GnRH circuits and/or pituitary functions, while *kiss1* is possibly implicated in the perception of environmental and metabolic signals.

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