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## Gonadotropin-inhibitory hormone in teleosts: New insights from a basal representative, the eel

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- 26 • The *gnih* gene encodes three GnIH peptides in a basal teleost, the European eel.
- 27 • *Gnih* mRNA is mostly expressed in the diencephalon in the eel.
- 28 • Eel GnIH peptides inhibit the expression of gonadotropin subunits *lhβ*, *fshβ*, *gpα* and  
29 of GnRH receptor, *gnrh-r2*, by eel primary cultures of pituitary cells.

30

### 31 **Abstract**

32 Since its discovery in birds, gonadotropin-inhibitory hormone (GnIH) has triggered  
33 investigation in the other groups of vertebrates. In the present study, we have identified a  
34 single *gnih* gene in the European eel (*Anguilla anguilla*), a representative species of a basal  
35 group of teleosts (Elopomorphs). We have also retrieved a single *gnih* gene in  
36 Osteoglossomorphs, as well as in more recently emerged teleosts, Clupeocephala. Phylogeny  
37 and synteny analyses allowed us to infer that one of the two *gnih* paralogs emerged from the  
38 teleost-specific whole genome duplication (TWGD or 3R), would have been lost shortly after  
39 the 3R, before the emergence of the basal groups of teleosts. This led to the presence of a  
40 single *gnih* in extant teleosts as in other vertebrates. Two *gnih* paralogs were still found in  
41 some teleost species, such as in salmonids, but resulting from the additional whole genome  
42 duplication that specifically occurred in this lineage (4R). Eel *gnih* was mostly expressed in  
43 the diencephalon part of the brain, as analyzed by quantitative real-time PCR. Cloning of eel  
44 *gnih* cDNA confirmed that the sequence of the GnIH precursor encoding three putative  
45 mature GnIH peptides (aaGnIH-1, aaGnIH-2 and aaGnIH-3), which were synthesized and  
46 tested for their direct effects on eel pituitary cells *in vitro*. Eel GnIH peptides inhibited the  
47 expression of gonadotropin subunits (*lhβ*, *fshβ*, and common *a*-subunit) as well as of GnRH  
48 receptor (*gnrh-r2*), with no effect on *tshβ* and *gh* expression. The inhibitory effect of GnIH  
49 peptides on gonadotropic function in a basal teleost is in agreement with an ancestral  
50 inhibitory role of GnIH in the neuroendocrine control of reproduction in vertebrates.

51

52

## 53 **1. Introduction**

54         The primary factor involved in the brain control of gonadotropin synthesis and  
55 secretion in vertebrates is the stimulatory decapeptide gonadotropin-releasing hormone  
56 (GnRH). Even if dopamine was reported to be a key inhibitor of gonadotropins in several  
57 teleost groups [for reviews: (Dufour et al., 2010; Peter et al., 1986)], no major peptidic  
58 inhibitory factor was identified in vertebrates until 2000. At that time, Tsutsui and  
59 collaborators described, from quail brain, the isolation and characterization of a novel peptide,  
60 having a RFamide (RFa) motif at the C-terminal extremity and inhibiting gonadotropin  
61 release from cultured quail anterior pituitaries, which they named gonadotropin-inhibitory  
62 hormone (GnIH) (Tsutsui et al., 2000). *Gnih* gene (also called *npvf* gene) homologs have been  
63 identified in non-avian vertebrates, including mammals, amphibians, fish and agnathans [for  
64 reviews: (Muñoz-Cueto et al., 2017; Ogawa and Parhar, 2014; Osugi et al., 2014b; Tsutsui et  
65 al., 2018)]. Multiple peptides are encoded by the *gnih* gene: two in mammals (RFRP-1 and  
66 RFRP-3), three in birds and reptiles (GnIH and two GnIH-related peptides, GnIH-RP1 and  
67 GnIH-RP2), four in amphibians (fGRP, fGRP-RP-1, fGRP-RP-2 and fGRP-RP-3 in the  
68 bullfrog, *Rana catesbeiana* and LPXRFa-1, -2, -3, -4 in the newt, *Cynops pyrrhogaster*) and  
69 two or three peptides in teleosts (LPXRFa or GnIHs) [for reviews: (Muñoz-Cueto et al., 2017;  
70 Ogawa and Parhar, 2014; Osugi et al., 2014b; Tsutsui et al., 2018; Ubuka and Parhar, 2018)].

71         A gene encoding three PQRf-peptides was identified in amphioxus (*Branchiostoma*  
72 *floridae*), representative of cephalochordates, the most basal group of chordates (Osugi et al.,  
73 2014a; Putnam et al., 2008). Phylogeny and synteny analyses indicate that this gene may  
74 represent the common ancestor to both *gnih* and *npff* (neuropeptide FF) genes, which would  
75 have arisen through the whole genome duplications that occurred in early vertebrates [(Osugi  
76 et al., 2014a); for reviews (Osugi et al., 2015; Tsutsui et al., 2018)].

77 *In vitro* studies of the direct pituitary effect of GnIH on gonadotropins have been  
78 performed in different vertebrate groups and gave contradictory results. While in birds, the  
79 direct inhibitory effect of GnIH on basal gonadotropin release, firstly reported in quail  
80 (Tsutsui et al., 2000), has been confirmed in chicken (Ciccione et al., 2004; Maddineni et al.,  
81 2008), in other vertebrates, the situation is different. In various mammals, RFRP-3 was only  
82 able to inhibit GnRH-induced, but not basal, luteinizing hormone (LH) and follicle-  
83 stimulating hormone (FSH) secretion and their expression by primary cultures of pituitary  
84 cells [ewe: (Clarke et al., 2008; Sari et al., 2009); bovine: (Kadokawa et al., 2009); pig: (Li et  
85 al., 2013)]. In rat, an inhibitory effect of RFRP-3 was observed *in vitro* on both basal and  
86 GnRH-stimulated LH secretion (Pineda et al., 2010), while other studies reported either no  
87 effect (Anderson et al., 2009) or only inhibition of GnRH-stimulated LH release (Murakami  
88 et al., 2008). In the Syrian hamster (*Mesocricetus auratus*), RFRP-3 had no effect *in vitro* on  
89 either basal nor stimulated LH release (Ancel et al., 2012). **It can be noticed that *in vivo***  
90 **studies showed a rapid inhibitory effect of GnIH/RFRP-3 on plasma LH levels in hamster**  
91 **(Kriegsfeld et al., 2006) as well as in rat (Rizwan et al., 2009), that was likely to be directly**  
92 **on the pituitary given the time-frame of the effect.** In amphibians, up to now, fGRP and its  
93 related peptides have been shown to exhibit *in vitro* a growth hormone (GH)-releasing  
94 activity, with no action on gonadotropin release (Koda et al., 2002; Ukena et al., 2003). In  
95 fish, the situation is even more complex. Besides the “classical” inhibitory action of GnIH  
96 like in birds, a stimulatory effect of GnIH on LH release *in vitro* was also reported in some  
97 teleost species [sockeye salmon, *Oncorhynchus nerka* (Amano et al., 2006); grass puffer,  
98 *Takifugu niphobles* (Shahjahan et al., 2011); tilapia, *Oreochromis niloticus* (Biran et al.,  
99 2014); catla, *Catla catla* (Kumar et al., 2019); for reviews: (Muñoz-Cueto et al., 2017; Ubuka  
100 and Parhar, 2018)]. In the goldfish *Carassius auratus*, using primary cultures of pituitary  
101 cells, Qi and collaborators have recently reported an inhibition of GnRH-stimulated *lhβ* and

102 *fsh $\beta$*  expression, with no effect on basal gonadotropin expression (Qi et al., 2013), while other  
103 studies reported either stimulatory, inhibitory or no effect on both basal and stimulated LH  
104 (release and expression) depending on the gonadal stage (Moussavi et al., 2013, 2012).

105 In the European eel (*Anguilla anguilla*), a member of elopomorphs, the most ancient  
106 group of teleosts (Chen et al., 2014; Henkel et al., 2012a), we have recently described an  
107 unexpected inhibitory effect of kisspeptin on *lh $\beta$*  expression by primary cultures of pituitary  
108 cells (Pasquier et al., 2018, 2011). This latter result raised the question whether, in the eel,  
109 GnIH could possess an inhibitory effect on gonadotropins or a “paradoxal” stimulatory effect  
110 (as in sockeye salmon, grass puffer, tilapia and catla) to be opposed to the unexpected  
111 inhibitory effect of kisspeptin.

112 In the present study, we have identified a single *gnih* gene in the European eel genome  
113 encoding three putative GnIH peptides. Our evolutionary study by phylogeny and synteny  
114 questioned the impact of the teleost-specific whole genome duplication (TGWD or 3R) on  
115 *gnih* paralog number in teleosts. We have investigated *gnih* tissue distribution in the European  
116 eel. We have also synthesised the predicted European eel GnIH mature peptides (*A. anguilla*,  
117 aa; aaGnIH-1, aaGnIH-2 and aaGnIH-3) and tested their effects on pituitary hormone and  
118 receptor expressions using primary cultures of eel pituitary cells.

119

## 120 **2. Materials and Methods**

121

### 122 **2.1. Animals and tissue sampling**

123 Freshwater female European eels (*A. anguilla*) were at the prepubertal "silver" stage,  
124 which corresponds to the last continental phase of the eel life cycle, preceding the oceanic  
125 reproductive migration. Cloning, tissue distribution and primary cultures were performed  
126 using female silver eels purchased from Gebr. Dil import-export BV (Akersloot, The

127 Netherlands) and transferred to MNHN, France. Animals were anesthetized by cooling and  
128 then killed by decapitation under the supervision of authorized person (KR; N°R-75UPMC-  
129 F1-08) according to the protocol approved by Cuvier Ethic Committee France (N°68-027).

130 Various tissues were collected for investigating the distribution of *gnih* expression  
131 (n=8 eels for each tissue). The following tissues were sampled, stored in RNAlater (Ambion  
132 Inc, Austin, TX, USA) and kept frozen at -20°C until RNA extraction: brain, pituitary, eye,  
133 gill, liver, kidney, intestine, spleen, muscle, adipose tissue (fat), and ovary. The brain was  
134 further dissected into six parts as previously described (Pasquier et al., 2018): olfactory bulbs,  
135 telencephalon, diencephalon, mesencephalon, *cerebellum* and *medulla oblongata*. For  
136 cloning, only the di-/mes-encephalon was dissected and used. For primary cultures, pituitaries  
137 were collected in culture medium just prior to dispersion (30 to 40 eels per cell culture).

138

## 139 **2.2. RNA extraction and cDNA synthesis**

140 Total RNA from tissue samples was extracted using Trizol reagent (Invitrogen, Cergy-  
141 Pontoise, France) according to the manufacturer's recommendations. Samples were  
142 homogenized by sonication a few seconds in Trizol. Following extraction, samples were  
143 submitted to a deoxyribonuclease I (Roche, Meylan, France) treatment. First strand cDNA  
144 was synthesized using 400 ng (tissue distribution) or 1 µg (cloning) of total RNA and a  
145 SuperScript III First Strand cDNA Synthesis Kit (Invitrogen), according to the manufacturer's  
146 recommendations. cDNAs were stored at -20°C until PCR and qPCR procedures.

147

## 148 **2.3. *In silico* prediction of European eel *gnih* gene and identification of vertebrate *gnih*** 149 **(*npvf*) genes**

150 *Gnih* gene was identified in the European eel genome assembly [GCA\_000695075.1;  
151 (Henkel et al., 2012b)] using TBLASTN search on CLC Workbench 6 (Qiagen, Vento, The  
152 Netherlands).

153 *Gnih* (*npvf*) gene sequences were sought in different vertebrates with a focus on teleost  
154 representatives. Gene sequences were retrieved from GenBank genomic database by an  
155 extensive Blast search using related species Gnih (NPVF) amino-acid sequences as query  
156 using CLC-Main Workbench browser. The annotation of non-annotated *gnih* (*npvf*) genes  
157 were performed either manually using the Blast alignment of the genomic sequence carrying  
158 the *gnih* (*npvf*) with protein sequences of related species *gnih* (*npvf*) or using the  
159 WebAUGUSTUS gene prediction server (<http://bioinf.uni-greifswald.de/augustus/>) with the  
160 *Danio rerio* parameters. All the sequence references as well as the genome assembly  
161 information are given in Supplementary Table 1. The nomenclature followed the  
162 recommended nomenclature in fish: the gene symbol is written in small letter in italic and the  
163 protein in capitals.

164

#### 165 **2.4. Cloning of eel *gnih* cDNA**

166 Specific cloning primer sets were designed, based on European eel *gnih* genomic  
167 sequence using Primer3 web browser (Supplementary Table 2). Three primer sets were  
168 designed 150-300 bp apart from the coding sequence while the fourth one flanked the coding  
169 sequence and was used for the nested PCR (Supplementary Table 2). All primers were  
170 purchased from Eurofins (Ebersberg, Germany).

171 PCR reactions were performed on a MyCycler Thermal Cycler (Bio-Rad, Marne-la-  
172 Coquette, France) in a final volume of 50 µl using GoTaq mix (Promega, Charbonnières,  
173 France) on brain cDNA. First PCR amplifications were carried out using the 3 first primer  
174 sets with the following thermal conditions after an initial step of 94°C for 2 min, 45 cycles at



175 94°C for 30 s, 56°C for 30 s and 72°C for 70 s and a final extension at 72°C for 5 min. The  
176 resulting PCR products were amplified in a nested PCR using the primer set 4 (F4R4) with  
177 higher stringent thermal conditions: an initial step of 94°C for 2 min, 45 cycles at 94°C for 30  
178 s, annealing temperature for 30 s, decreasing every 5 cycles from 60°C to 57.5°C, 72°C for  
179 70s, and a final extension at 72°C for 5 min. Amplified products were separated on agarose  
180 gel and purified using QIAquick gel extraction kit (Qiagen). Purified PCR fragments were  
181 sequenced after subcloning into a TOPO cloning vector (Invitrogen), by the sequencing  
182 service of GATC Biotech (Constance, Germany).

183

## 184 **2.5. Phylogeny analysis**

185 Multiple sequence alignments of amino-acid sequence of GnIH (NPVF)  
186 preproteins were performed using the progressive alignment algorithm available on CLC  
187 workbench and further manually edited. The tree topology was inferred using Maximum  
188 likelihood algorithm using PhyML 3.0 and with JTT as substitution model on SeaView 4.64  
189 (Gouy et al., 2010). Strength of branch nodes was first evaluated by aLRT test and then by  
190 bootstrap using 100 replicates. The consensus tree was plotted using the R package tree (Yu et  
191 al., 2017). The GnIH (LPXRF) of lamprey (*Petromyzon marinus*) and the PQRF of  
192 amphioxus were used to root the tree.

193

## 194 **2.6. Synteny analysis**

195 The genomic region carrying the *gnih/npvf* of the spotted gar (*Lepisosteus oculatus*),  
196 as an actinopterygian non-teleostean, was considered as reference region before the 3R  
197 duplication. The 3R duplicates of the *gnih/npvf* flanking genes were sought in the genome of  
198 teleost representatives, focusing on the European eel (*A. anguilla*) and the Japanese eel  
199 (*Anguilla japonica*), and other basal teleost species, an osteoglossomopha, the arowana

200 (*Scleropages formosus*) and an ostariophysi, the zebrafish (*Danio rerio*). The 3R-paralogous  
201 neighbouring genes were mapped by blast search and by using gene prediction lists of the  
202 genomic region identified as being the corresponding 3R-ohnologous regions. For the eel, the  
203 genes of the two genomic 3R-ohnologous regions were predicted using Augustus prediction  
204 server. References and locations of the genes of the synteny analysis are given in the  
205 Supplementary Table 3.

206

## 207 **2.7. Sequence predictions, synthesis and 3D structure predictions of eel GnIH peptides**

208 Mature peptides were predicted from the eel GnIH precursor using NeuroPred web  
209 browser [<http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py>, (Southey et al., 2006)].  
210 Signal peptide of GnIH precursors were predicted using Signal 4 (Petersen et al., 2011) on  
211 CLC workbench.

212 Predicted European eel GnIH peptides [aaGnIH-1 (19 aa), aaGnIH-2 (20 aa) and  
213 aaGnIH-3 (32 aa); Table 1] were synthesized as previously described (Gutierrez-Pascual et  
214 al., 2009; Pasquier et al., 2018).

215 Structure predictions of eel GnIH peptides were modelled using the I-TASSER server,  
216 an automated protein-modeling server (Yang et al., 2014). Only models with the C-score  
217 between 2 and -5 were considered. Visualization of the structures was performed using the  
218 Jmol software v2.1 (Jmol: an open-source Java viewer for chemical structures in 3D.  
219 <http://www.jmol.org/>).

220

## 221 **2.8. Primary culture of eel pituitary cells**

### 222 **2.8.1. Dispersion and culture**

223 Dispersion and primary culture of pituitary cells were performed using an enzymatic  
224 and mechanical method as previously described (Montero et al., 1996). Briefly, pituitaries

225 were cut into slices and incubated at 25°C in a solution of porcine type II trypsin (Sigma-  
226 Aldrich, Saint-Quentin Fallavier, France). After 1 h, the trypsin solution was replaced by a  
227 solution of DNase (Sigma-Aldrich) and soya bean trypsin inhibitor (Sigma-Aldrich) for  
228 10min. Pituitary slices were then washed with calcium-free phosphate-buffered saline (PBS)  
229 (Gibco, Thermo Fisher scientific, Villebon-sur-Yvette, France) and mechanically dispersed by  
230 repeated passages through a plastic transfer pipette (Falcon, Thermo Fisher scientific,  
231 Villebon-sur-Yvette, France). After estimating the number of viable cells by Trypan Blue  
232 coloration (Sigma-Aldrich), cells were plated on 96-well plates (60,000 cells/well) pre-coated  
233 with poly-L-lysine (Sigma-Aldrich). Cultures were performed in serum-free culture medium  
234 (Medium 199 with Earle's salt and sodium bicarbonate buffer, 100 U/ml penicillin, 100 µg/ml  
235 streptomycin, 250 ng/ml fungizone; Gibco) at 18°C under 3% CO<sub>2</sub> and saturated humidity.

### 236 **2.8.2. *In vitro* treatments**

237 GnIH treatments were started 24 h after the beginning of culture to allow cell  
238 attachment (Day 0). Replicates of 5 wells for control and each treated group were used. Eel  
239 GnIH peptides (sequences are given in Table 1) stock solutions (10<sup>-4</sup> M) were prepared in  
240 ultrapure water and stored at -20°C. Stock solutions were diluted in culture medium just  
241 before addition to the culture wells. Culture medium was changed and treatment added to the  
242 cells on Day 0, Day 3, Day 7 and Day 10. Cultures were stopped on Day 10. The effects of  
243 treatments were tested in 3 to 6 independent experiments performed on different cell  
244 preparations from different batches of fish. Figures display the pooled results of these  
245 different experiments.

### 246 **2.8.3. Cell RNA extraction and cDNA synthesis**

247 Total RNA was directly extracted in wells using the Cell-to-cDNA II Kit (Ambion  
248 Inc.) according to the manufacturer's recommendations. Cells were washed with PBS (Gibco)  
249 and lysed with Cell Lysis II Buffer (80 µl/well). The lysates were digested with RNase-free

250 DNase I (Roche). Four  $\mu$ l of RNA solution of each samples was then reverse transcribed with  
251 a SuperScript III First Strand cDNA Synthesis Kit (Invitrogen). The samples obtained were  
252 stored at  $-20^{\circ}\text{C}$  until qPCR.

253

## 254 **2.9. Quantitative real-time PCR (qPCR)**

### 255 **2.9.1. Primers**

#### 256 **2.9.1.1. Design of primers for eel *gnih***

257 Eel *gnih* specific primers (Supplementary Table 2) were designed based on the European eel  
258 *gnih* sequence obtained in this study, using the Primer3 Software (Whitehead  
259 Institute/Massachusetts Institute of Technology, Boston, MA). Amplicon size was 125 bp.  
260 Forward and reverse primers were located in different exons to prevent amplification of  
261 genomic DNA. To optimize the assay, different annealing temperature were tested according  
262 to the  $T_m$  of primers. To check their specificity, amplification products were sequenced at  
263 GATC biotech.

#### 264 **2.9.1.2. Other gene primers**

265 Gene specific primers were previously designed based on the nucleotide sequence of  
266 the European eel *gpa* (Quérat et al., 1990a), *fsh $\beta$*  (Schmitz et al., 2005), *lh $\beta$*  (Quérat et al.,  
267 1990b), *gh* (Gong et al., 2002), thyrotropin- $\beta$  subunit [*tsh $\beta$* ; (Maugars et al., 2014)] GnRH-  
268 type 2 receptor [*gnrh-r2*; (Peñaranda et al., 2013)] and  *$\beta$ -actin* (Aroua et al., 2007) cDNA, the  
269 latter being used as reference gene. European eel possesses, as other teleosts, a dual TSH  
270 system [European eel (Maugars et al., 2014); Atlantic salmon *Salmo salar* (Fleming et al.,  
271 2019)]: a “classical” *tsh $\beta$*  (named *tsh $\beta$ 1* or *tsh $\beta$ a*), which is exclusively expressed in the  
272 pituitary (and that was assayed in this study) and a second *tsh $\beta$*  (named *tsh $\beta$ 3* or *tsh $\beta$ b*) with  
273 low expression in pituitary and high expression in ovary (Maugars et al., 2014). European eel  
274 possesses three GnRH receptors (GnRH-R1a, GnRH-R1b and GnRH-R2) and GnRH-R2 is

275 the only one whose expression increases during female maturation (Peñaranda et al., 2013).  
276 *Gnrh-r1a* and *gnrh-r1b* expressions were below the threshold of detection in primary cultures  
277 of pituitary cells (Campo et al., 2018; Pasquier et al., 2018), and were thus not assayed in this  
278 study.

279 The qPCR primers are listed in Supplementary Table 2. They were all purchased from  
280 Eurofins.

281

### 282 **2.9.2. Quantitative PCR assays**

283 Quantitative PCR assays were performed using the LightCycler® System (Roche, Ltd.  
284 Basel, Switzerland) with SYBR Green I sequence-unspecific detection as previously  
285 described (Campo et al., 2018; Pasquier et al., 2018, 2011). The qPCRs were prepared with 4  
286  $\mu$ l of diluted cDNA template, 2  $\mu$ l of PCR grade water, 2  $\mu$ l of SYBR Green master mix and 1  
287  $\mu$ l of each forward and reverse primer (500 nM each at final concentration). The protocol was  
288 an initial step of polymerase activation for 10 min at 95°C; then 41 cycles ( *$\beta$ -actin*, *gh*, *gp $\alpha$* ,  
289 *lh $\beta$* , *fsh $\beta$*  and *tsh $\beta$* ) of 10 s at 95°C for denaturing, 5 s at 60°C for annealing, 10 s at 72°C for  
290 primer extension and a single final extension step of 5 min at 72°C. For *gnih*, the protocol was  
291 an initial step of polymerase activation for 10 min at 95°C; 41 cycles of 15 s at 95°C, 5 s at  
292 60°C, 10 s at 72°C and a single final extension step of 5 min at 83°C. For *gnrh-r2*, the protocol  
293 was an initial step of polymerase activation for 10 min at 95°C; 42 cycles of 10 s at 95°C, 7 s  
294 at 61°C, 4 s at 72°C and a single final extension step of 5 min at 72°C. Each program ended  
295 with a melting curve analysis by slowly increasing the temperature (0.01°C/s) up to 95°C with  
296 a continuous registration of changes in fluorescent emission intensity. Serial dilutions of  
297 cDNA pools of either brain (*gnih* tissue distribution) or pituitary cells (cell culture  
298 experiments) were used as a standard curve. One chosen dilution was also included in each  
299 run as a calibrator. Each qPCR run contained a non-template control (cDNA was substituted

300 by water) for each primer pairs to confirm that reagents were not contaminated. The  
301 efficiency of all primers was tested, and the specificity of each reaction was assessed by  
302 melting curve analysis to ensure the presence of only one product. Each sample was analyzed  
303 in duplicate by qPCR. Normalisation of data was performed using total RNA content for the  
304 tissue distribution samples, and using *β-actin* mRNA level for cell culture samples.

305

## 306 **2.10. Statistical analysis**

307 Results are given as mean ± SEM. Means were compared by one-way ANOVA  
308 Tukey's multiple comparison test using InStat (GraphPad Software Inc., San Diego, Calif.,  
309 USA).

310

## 311 **3. Results**

### 312 **3.1. Prediction of European eel *gnih* gene**

313 A single *gnih* (*npvf*) gene was found in European eel genome [on the scaffold  
314 AZBK01S009502.1 of the *Anguilla anguilla*\_v1\_09\_nov\_10 genome assembly and on the  
315 scaffold 1747 of the Racon- and Pilon-corrected candidate assembly; (Jansen et al., 2017)]. A  
316 single *gnih* gene was also identified in the American eel *Anguilla rostrata* (assembly  
317 ASM160608v1) and the Japanese eel *A. japonica* (on the scaffold BEWY01082450 of the  
318 Ajp\_01 assembly) genomes. The *gnih* (*npvf*) gene found in Japanese eel genome  
319 corresponded to the *gnih* (*npvf*) nucleic sequence (LC163968).

320

### 321 **3.2. Cloning of cDNA**

322 Using European eel specific *gnih* primers, PCRs performed on brain cDNAs led to the  
323 identification of a 654 bp Gnih transcript sequence (GenBank: MN022784) corresponding

324 the full-length coding sequence (CDS). Once translated, the cloned GnIH CDS gave a 217-aa  
325 GnIH precursor (Supplementary Figure 1).

326 The comparison of *gnih* mRNA to the European eel genome allowed to reannotate the  
327 *GnIH* genomic sequence (Supplementary Figure 2) and showed that the transcript is encoded  
328 by 3 exons. Exon 1 (141 bp) encoded for N-terminal region (52aa) including the signal  
329 peptide in N-terminal position. Exon 2 (410 bp) encoded for the 3 GnIH peptides (137 aa).  
330 The cloned sequence was identical to the corresponding sequence in the European eel  
331 genome.

332

### 333 **3.3. Identification of *gnih* (*npvf*) genes and phylogeny analysis**

334 We have investigated the *gnih* (*npvf*) in genome assemblies, recently released, of key  
335 teleost species including representatives of two basal groups, Elopomorphs, with the Japanese  
336 eel (*A. japonica*) and the European eel (*A. anguilla*), and Osteoglossomorphs, with the  
337 arowana (*S. formosus*), the arapaima (*Arapaima gigas*), and the *Paramormyrops kingsleyae*.  
338 A single *gnih* gene was found in the genomes of the basal teleosts, elopomorphs and  
339 osteoglossomorphs, as well as in the genomes of the other teleost species examined, except  
340 for the salmonid species where two *gnih* genes were identified (Supplementary Figure 3). In  
341 the Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) two *gnih* genes were found on  
342 two different chromosomes (Ssa14 and Ssa27 in salmon genome assembly ICSASG\_v2 and  
343 on the Chr14 and Chr18 in trout genome assembly Omyk\_1.0), likely as a result of the  
344 salmonid-specific genome duplication (4R).

345 In the attempt to identify *gnih* (*npvf*) genes in representatives of all vertebrate classes,  
346 we have also searched for *gnih* in chondrichthyans, using new released genomes: in bamboo  
347 shark (*Chiloscyllium punctatum*, Cpunctatum v1\_0), cloudy catshark (*Scyliorhinus torazame*,  
348 Storazame\_v1.0) and whale shark (*Rhincodon typus*, ASM164234v2). No *gnih* (*npvf*) was

349 found in these three chondrichthyans. In contrast, in these three species, blast search returned a  
350 gene encoding NPPF, the sister gene of *gnih* (*npvf*) in vertebrates.

351 The phylogeny analysis was conducted on 33 GnIH (NPVF) preproproteins from 31  
352 osteichthyan species including 20 teleosts, and using the preproGnIH (LPXRF) of lamprey  
353 and the preproPQRF of amphioxus as outgroup (Figure 1). All osteichthyan preproGnIH  
354 (preproNPVF) sequences clustered into a single clade. Coelacanth (*Latimeria chalumnae*)  
355 preproGnIH sequence was at the basis of the other sarcopterygian sequences, in agreement  
356 with its phylogenetical position. All teleost preproGnIH sequences formed a single clade. The  
357 position of the preproGnIH sequence of a non-teleost actinopterygian, at the basis of the  
358 sarcopterygian clade rather than at the basis of the teleost clade was unexpected, and may  
359 reflect a specific divergence of teleost sequences. Among the teleost clade, the elopomorph  
360 European eel and Japanese eel preproGnIH sequences clustered together within a clade  
361 grouping also the three osteoglossomorph species. The sequences from the other teleosts  
362 grouped in another clade, with a tree typology in agreement with their phylogenetic positions.  
363 The duplicated GnIH of salmonids, rainbow trout and Atlantic salmon, were illustrated. Each  
364 of the duplicated salmonid preproGnIH clustered with one of the duplicated trout  
365 preproGnIH, reflecting the common 4R-salmonid origin of the duplicated paralogs. The  
366 single preproGnIH of the pike, *Esox lucius*, representative of the esociforms, sister group of  
367 salmonids but which has not undergone the 4R, was at the basis of the salmonid duplicated  
368 sequences, in agreement with the phylogeny.

369

### 370 **3.4. Synteny analysis**

371 We have retrieved only a single *gnih* (*npvf*) gene in basal teleosts, elopomorphs and  
372 osteoglossomorphs, as in the other groups of teleosts, more recently emerged (clupeocephala).  
373 In order to elucidate the question of the fate of the *gnih* duplicated paralogs after the teleost



374 3R, and of the orthology between the single *gnih* of basal teleosts and the single *gnih (npvf)* of  
375 clupeocephala, we have performed a synteny analysis (Figure 2). The *gnih (npvf)*  
376 neighbouring genes were studied in the non-teleost actinopterygian, the spotted gar, as a  
377 reference and in representative teleosts, including basal species such as the eels and the  
378 arowana (Figure 2), and clupeocephala such as the zebrafish, pike, and tilapia. The synteny  
379 analysis confirmed the duplication of the *gnih* genomic region, in agreement with the 3R.  
380 *Gnih* neighbouring genes in spotted gar such as *mpp6*, *cbx3*, *snx10*, *hox9* were duplicated and  
381 conserved on each 3R-duplicated genomic region in all teleosts studied in the synteny  
382 analysis. In contrast, a single 3R-paralog was conserved for *gnih*, as well as for some  
383 neighbouring genes, such as *pde11a*, *nfe2l3* and *skap2* in all teleost species studied.  
384 Remarkably, for all teleosts studied, the single *gnih* paralog was found within the same 3R-  
385 duplicated genomic region, reflecting the orthology of the conserved single *gnih* paralog of  
386 basal teleosts and clupeocephala. The teleost *gnih* genomic region is indeed characterized by  
387 the presence of the single conserved *nfe2l3* paralog, in adjacent position to *gnih*, and the loss  
388 of *pde11a* and *skap2* paralogs, which have been conserved only by the other 3R-duplicated  
389 genomic region.

390

### 391 **3.5. Tissue distribution of European eel *gnih* mRNA**

392 Specific qPCR protocol was developed for eel *gnih* and applied to the analysis of the  
393 tissue distribution of its transcripts (Figure 4). *Gnih* mRNA was specifically expressed in the  
394 diencephalon part of the brain. Its expression was close to the limit of detection in the other  
395 parts of the brain (olfactory bulbs, telencephalon, mesencephalon, *cerebellum* and *medulla*  
396 *oblongata*), as well as in pituitary and peripheral tissues (Figure 3).

397

### 398 **3.6. Prediction and three-dimensional structure of European eel mature GnIH peptides**

399           The identification of the potential N-terminal and C-terminal cleavage sites led to the  
400 prediction of three mature peptides (aaGnIH-1, aaGnIH-2 and aaGnIH-3) (Table 1). Two of  
401 them present the conserved domain of LPXRF peptides, with the 19-amino-acid aaGnIH-1  
402 ending by the LPLRF motif, and the 32-amino acid aaGnIH-3 ending by the LPQRF motif. In  
403 contrast, aaGnIH-2, a 20-amino-acid peptide, exhibited a LTTRF C-terminal extremity. All  
404 the three predicted peptides were amidated due to the presence of a GR site downstream their  
405 position in the GnIH precursor.

406           GnIH-1 peptide sequence was the same in the three *Anguilla* species investigated (*A.*  
407 *anguilla*, *A. rostrata* and *A. japonica*). GnIH-2 and -3 sequences were also identical in *A.*  
408 *rostrata* and *A. anguilla*, but in *A. japonica*, GnIH-2 peptide differed by two amino-acids  
409 (Ala-Gln instead of Tyr-Arg in positions 7-8) and GnIH-3 peptide by one amino-acid (Ile  
410 instead of Met in position 18). A third amino-acid (Ser instead of Pro in position 3) differed in  
411 *A. japonica* cloned cDNA (BAV18007.1) but not in the genomic sequence (Table 1;  
412 Supplementary Figure 3).

413           The three-dimensional (3D) secondary structures of the three European eel GnIH  
414 peptides were predicted using the I-TASSER server (Figure 4). The 3 peptides had a similar  
415 C-terminal coiled region, and they presented an internal  $\alpha$ -helix with some differences in the  
416 size and location. For aaGnIH-1, the helix size was 7 amino acids (residues 8 to 14) and 8  
417 amino acids for aaGnIH-3 (residues 12 to 19). Their helices were in the central part of the  
418 peptide and their N- and C-terminal extremities were close spatially, or even nested for  
419 aaGnIH-3. On the other hand, aaGnIH-2 had a longer helix (11 residues from 2 to 12) that  
420 started very close to the N-terminal extremity of the peptide and had an extended overall  
421 conformation.

422

423 **3.7. *In vitro* effects of synthesized eel GnIH peptides on the expression of pituitary**  
424 **hormones by primary cultures of eel pituitary cells**

425 The three predicted European eel mature GnIH peptides (aaGnIH-1, -2 and -3) were  
426 synthesized and their effects tested on 10-day primary cultures of eel pituitary cells for 10  
427 days, as previously performed with eel kisspeptin and tachykinin peptides (Campo et al.,  
428 2018; Pasquier et al., 2018).

429 **3.7.1. Dose-dependent effect of eel GnIH peptides on *lhβ* mRNA levels**

430 The effects of various concentrations of aaGnIH-1, aaGnIH-2 and aaGnIH-3 (from 10<sup>-</sup>  
431 <sup>10</sup> to 10<sup>-6</sup> M) were tested over 10 days of culture (Figure 5A). None of the three eel GnIH  
432 peptides had significant effect on *lhβ* mRNA levels at 10<sup>-10</sup> M. In contrast, at 10<sup>-8</sup> and 10<sup>-6</sup> M,  
433 they all significantly and dose-dependently inhibited *lhβ* mRNA levels in comparison to  
434 controls (aaGnIH-1: P < 0.001 at both doses; aaGnIH-2: P<0.05 at 10<sup>-8</sup> M and P<0.001 at 10<sup>-6</sup>  
435 M; aaGnIH-3: P < 0.001 at both doses). None of the three eel GnIH at any dose tested  
436 affected the mRNA levels of *β-actin* (Figure 5B).

437 **3.7.2. Effects of eel GnIH peptides on other pituitary hormone and receptor mRNA**  
438 **levels**

439 To test whether the inhibitory effect of eel GnIHs was specific to *lhβ* expression, the  
440 effect of 10<sup>-6</sup> M aaGnIH-1, aaGnIH-2 and aaGnIH-3 was investigated on other pituitary  
441 hormones (Figure 6) over 10 days. As in the experiment above, all three eel GnIH peptides  
442 significantly inhibited *lhβ* mRNA levels (54, 33 and 53% of inhibition, respectively, P <  
443 0.001). Eel GnIH-2 and -3 peptides slightly but significantly inhibited *fshβ* mRNA levels (17  
444 and 11 % of inhibition, respectively; P<0.05). All three aaGnIH peptides also inhibited  
445 common α glycoprotein subunit (*gpα*) mRNA levels (31, 18 and 33% of inhibition,  
446 respectively; P < 0.001 for aaGnIH-1 and -3; P<0.05 for aaGnIH-2). Eel GnIH peptides also  
447 inhibited pituitary GnRH receptor, *gnrh-r2*, mRNA levels (50, 40 and 38% of inhibition,

448 respectively;  $P < 0.001$ ). In contrast, the peptides had no effect on other pituitary hormone,  
449 *tsh $\beta$*  and *gh*, mRNA levels (Figure 6).

450

## 451 **4. Discussion**

452

### 453 **4.1. Characterization of European eel *GnIH***

454 A single *gnih* gene was identified in the genome of the European eel, one  
455 representative of elopomorphs, as in other teleosts and in tetrapods. Using NeuroPred  
456 software, three mature peptides of 19 (aaGnIH-1), 20 (aaGnIH-2) and 32 (aaGnIH-3) amino-  
457 acids were predicted in the European eel. A single *gnih* gene encoding three mature peptides  
458 was also found in other *Anguilla* species. Three putative peptides are also encoded in the  
459 GnIH precursor of an osteoglossomorph (arowana), some cypriniformes (goldfish, common  
460 carp *Cyprinus carpio*, zebrafish, catla, red piranha *Pygocentrus nattereri*, golden-line barbel  
461 *Sinocyclocheilus grahami* and *Sinocyclocheilus rhinoceros*), a siluriforme, the catfish  
462 (*Ictalurus punctatus*); a salmoniforme, (Atlantic salmon) and some percomorpha (orange-  
463 spotted grouper *Epinephelus coioides*, Senegalese sole *Solea senegalensis*, Japanese flounder,  
464 *Cichlasoma dimerus*, tilapia, medaka *Oryzias latipes* and, bluehead wrasse *Thalassoma*  
465 *bifasciatum*) [for reviews: (Di Yorio et al., 2019; Muñoz-Cueto et al., 2017)], as in most  
466 tetrapods [birds and reptiles; for reviews: (Ogawa and Parhar, 2014; Osugi et al., 2014b;  
467 Tsutsui et al., 2018)]. In some other teleosts, belonging to the superorder Acanthopterygi,  
468 only two peptides are predicted from GnIH precursor [for reviews: (Di Yorio et al., 2019;  
469 Muñoz-Cueto et al., 2017)]. Thus, in teleosts, only late-branching evolved species exhibit two  
470 encoded peptides, suggesting a loss of one peptide in the course of teleost evolution (Di Yorio  
471 et al., 2019). The existence of only two peptides in the GnIH precursor is also observed in  
472 mammals [for reviews: (Ogawa and Parhar, 2014; Osugi et al., 2014b; Tsutsui et al., 2018)].

473 The multiple sequence alignment of the GnIH preproteins highlighted that the spotted gar,  
474 a non-teleostean actinopterygian, presents three putative functional LPXRFa peptides and the  
475 stigmat of a fourth at the C-terminal extremity, matching with the fGRP-RP-3-like of the  
476 frogs and the GRP-RP-2-like of the lizards and the GnIH-RP-2 of the chicken (Supplementary  
477 Figure 3). In coelacanth, five LPXRFa peptides could be predicted, the four first  
478 peptides correspond to the four peptides predicted in frog and in lizard, while the fifth found  
479 at the C-terminal extremity appeared to be specific of the coelacanth.

480 European eel peptides, aaGnIH-1 and -3, possessed the conserved C-terminal LPXRF  
481 motif (LPLRFamide and LPQRFamide, respectively), followed by an amidation site (Gly-  
482 Arg) common to most vertebrate GnIHs [for review: (Osugi et al., 2014b)]. In contrast,  
483 aaGnIH-2 possessed another RFamide signature, LTRRFamide, which is, to the best of our  
484 knowledge, unique among vertebrates/teleosts. The presence of a RFamide motif different  
485 from LPXRF at the C-terminal extremity of a predicted GnIH is also observed in some other  
486 teleosts. For instance, the signature MPXRF (X=M or Q) is found in all or some of the  
487 predicted GnIH peptides from takifugu/tiger puffer *Takifugu rubripes*, tetraodon *Tetraodon*  
488 *nigroviridis* and stickleback *Gasterosteus aculeatus* (Zhang et al., 2010), grass puffer  
489 (Shahjahan et al., 2011), medaka (Osugi et al., 2014b), tilapia (Biran et al., 2014), orange-  
490 spotted grouper (Wang et al., 2015), half-smooth tongue sole *Cynoglossus semilaevis* (Wang  
491 et al., 2018), Senegalese sole (Aliaga-Guerrero et al., 2018), European sea bass *Dicentrarchus*  
492 *labrax* (Paullada-Salmerón et al., 2016a), *Cichlasoma dimerus* (Di Yorio et al., 2016) [for  
493 reviews : (Di Yorio et al., 2019; Muñoz-Cueto et al., 2017)]. Thus, Muñoz-Cueto and  
494 collaborators chose to name these teleost peptides LPXRF-like peptides (Muñoz-Cueto et al.,  
495 2017). Such MPXRFa motif (X=L) is also observed in a bird (Pekin drake, *Anas*  
496 *platyrhynchos domestica*) for GnIH-RP1 (Fraley et al., 2013). In the red-eared slider turtle  
497 (*Trachemys scripta elegans*), in addition to three LPXRF (X=L or Q) peptides, another

498 RFamide (LLHRFamide) sequence is observed in the precursor between GnIH and GnIH-RP-  
499 2, leading to four potential RF-peptides (Ukena et al., 2016). In lamprey, the C-terminal  
500 sequence of LPXRFa-1 and -2 is QPQRFa, while LPXRFa-2-like peptide ends with RPQRFa  
501 (Osugi et al., 2012). All the above-mentioned peptides end by RFa, but in some teleosts, one  
502 of the predicted GnIH peptides is not an RF-amide: HQVRYa in grass and tiger puffer  
503 (Shahjahan et al., 2011) or LPQRLa in grouper (Wang et al., 2015). In the coelacanth, GnIH-  
504 2 also ends by LPLRLa (Muñoz-Cueto et al., 2017). The human GnIH precursor, in addition  
505 to RFRP-1 (-LPLRFamide) and RFRP-3 (-LPQRFamide), also encodes a predicted RFRP-  
506 like peptide with a C-terminus LPLRSamide motif (Ubuka et al., 2009). In tilapia, the third  
507 putative RFa-related peptides (RFRP) encoded by GnIH precursor lacks an amidation site  
508 (Biran et al., 2014). Further studies need to be done to investigate whether these non-  
509 conventional GnIH peptides, present in some vertebrates, are produced by the brain and  
510 possess physiological effects.

511

#### 512 **4.2. Expression of *gnih* mRNA in the diencephalon part of the brain**

513 The tissue distribution of *gnih* transcripts, which was analysed in the present study in  
514 females at silver stage, may differ in males or in females at different physiological stage.  
515 qPCR data showed a high and specific expression of *gnih* in the diencephalon part of the eel  
516 brain, in agreement with previous data in other vertebrates (for review: Ogawa and Parhar,  
517 2014). The intense presence of GnIH neurons in the diencephalic *nucleus posterioris*  
518 *periventricularis* (NPPv) was found to be a common pattern in teleosts [for reviews: (Di  
519 Yorio et al., 2019; Muñoz-Cueto et al., 2017)]. Projections of GnIH fibers to the pituitary  
520 have been reported in various teleost species, providing the anatomical basis for the  
521 neuroendocrine role of GnIH [for review: (Di Yorio et al., 2019)]. However, in some teleosts,  
522 no GnIH pituitary innervation was observed [Indian major carp, *Labeo rohita* (Biswas et al.,

523 2015); *Cichlasoma dimerus* (Di Yorio et al., 2016)]. Immunocytochemical studies of GnIH  
524 neurons in the eel would allow to assess the pituitary innervation, as well as possible brain  
525 projections linked to potential additional extra-pituitary functions of GnIH.

526 In the eel, *gnih* transcript levels were very low, at the limit of detection threshold, in  
527 other parts of the brain than diencephalon. This differs from some other neuropeptides such as  
528 kisspeptins (Pasquier et al., 2018) and tachykinins (Campo et al., 2018), which are more  
529 widely expressed in the eel brain. In some teleosts, such as Indian major carp (Biswas et al.,  
530 2015) and European sea bass (Paullada-Salmerón et al., 2016a) GnIH-immunoreactive (-ir)  
531 cells and *gnih* expression are observed in various brain regions, while in some other teleosts,  
532 GnIH-ir cells were restricted to NPPv [sockeye salmon (Amano et al., 2006); tilapia (Ogawa  
533 et al., 2016); for reviews: (Di Yorio et al., 2019; Muñoz-Cueto et al., 2017)]. This suggests  
534 variations among teleost species in the potential roles of GnIH as brain neuromediator.

535 In some teleosts, *gnih* expression has also been reported in peripheral tissues, in  
536 particular in gonads [for reviews: (Muñoz-Cueto et al., 2017; Ogawa and Parhar, 2014)]. Such  
537 a gonadal expression has also been reported in birds, mammals and primates, suggesting a  
538 role in steroid synthesis and gamete maturation [for review: (McGuire and Bentley, 2010)]. In  
539 the prepubertal female silver eel, the expression of GnIH was very low, at the limit of  
540 detection threshold in the ovary as well as in the other peripheral tissues investigated.

541

#### 542 **4.3. Direct pituitary inhibitory effect of eel GnIH peptides on gonadotropic axis**

543 We have shown that the predicted mature eel GnIH peptides possess a significant  
544 inhibitory effect on gonadotropin subunits, *lhβ*, *fshβ*, *gpα*, and GnRH receptor, *gnrh-r2*,  
545 mRNA expression by primary cultures of pituitary cells from prepubertal silver eels. Whether  
546 these decreases in mRNA expression involved a reduction in the relative number of  
547 gonadotrophs in the culture could deserve some future investigations. It should be also

548 noticed that these results have been obtained in females at silver stage, and may differ in  
549 males or in females at a different physiological stage. Our study focused on transcript levels  
550 and did not investigate gonadotropin release, as gonadotropin levels in the culture medium of  
551 silver eel pituitary cells are too low to be detected (Montero et al., 1996).

552 Inhibitory or stimulatory effects of GnIH peptides on *lhβ* expression *in vitro* have been  
553 reported in some other teleosts. Some experiments were also performed on primary cultures  
554 of pituitary cells. *In vitro* experiments in the goldfish show a seasonal variation of GnIH  
555 effect on *lhβ* expression by pituitary cells. Indeed, gfGnIH-3 (gfLPXRFa-3 or gGnIH)  
556 suppresses *lhβ* mRNA levels at early recrudescence and prespawning but elevated *lhβ* at mid-  
557 recrudescence (Moussavi et al., 2012). In contrast, Qi and collaborators reported no effect of  
558 gfGnIH-3 (GnIH-III) on basal gonadotropin synthesis, but an inhibition of GnRH-stimulated  
559 *lhβ* synthesis by primary culture of pituitary cells from female goldfish in the late vitellogenic  
560 stage (Qi et al., 2013). In their study, GnIH-2 (GnIH-II) has no effect neither on basal nor on  
561 stimulated *lhβ* expression (Qi et al., 2013). Various effects of GnIH were also reported in  
562 other teleosts. In grass puffer, goldfish (gf) LPXRFa stimulates *lhβ* mRNA expression in  
563 cultured pituitary cells [only gfLPXRFa-1 was tested because of its high sequence similarity  
564 to grass puffer LPXRFa-1 (Shahjahan et al., 2011)]. Others *in vitro* studies used whole  
565 (explants) or diced pituitaries, which could not discriminate between direct effects on  
566 pituitary cells or indirect effects on neurosecretory fibers innervating the pituitary cells.  
567 Indeed, in teleosts, differently from tetrapods, hypophysiotropic nerve endings directly  
568 innervate the adenohypophysis [for review: (Zohar et al., 2010)]. In common carp, GnIH-3  
569 (GnIH-III) decreases *lhβ* mRNA expression by diced pituitaries (Peng et al., 2016). In  
570 zebrafish, LPXRFa-3 reduces *lhβ* mRNA levels by adult male pituitary explants; the two  
571 other peptides encoded in the zebrafish LPXRFa precursor were not tested (Spicer et al.,  
572 2017). In *Astyanax altiparanae* males, zebrafish GnIH-3 attenuates the cGnRH2-stimulatory



573 action on *lhβ* mRNA expression by pituitary explants, without any effect on basal *lhβ*  
574 expression level (Branco et al., 2019). In contrast, Wang and collaborators describe a  
575 stimulation of basal *lhβ* mRNAs by LPXRFa-1 by whole pituitary culture in half-smooth  
576 tongue sole (Wang et al., 2019b). In chicken *Gallus gallus*, GnIH treatment of diced pituitary  
577 glands from adult cockerels has no effect on *lhβ* mRNAs (Ciccione et al., 2004).

578 In European eel, aaGnIH-2 and -3, but not aaGnIH-1, inhibited *fshβ* expression, while  
579 they all inhibited *lhβ* expression. This may implicate that different GnIH receptors [for now,  
580 up to 3 in teleosts (Wang et al., 2019a)] are present on pituitary *lhβ*- and *fshβ*- expressing  
581 cells, which should be further investigate in our model. In goldfish, Qi and collaborators  
582 report no effect on basal gonadotropin synthesis, but an inhibition of GnRH-stimulated *fshβ*  
583 synthesis by pituitary cells from female goldfish in the late vitellogenic stage treated with  
584 gfGnIH-III (Qi et al., 2013). Moussavi and collaborators have already demonstrated that  
585 gfLPXRFa-3 (gGnIH) consistently attenuates basal and GnRH-induced *fshβ* mRNA levels in  
586 a dose-dependent manner in mid and late gonadal recrudescence goldfish (Moussavi et al.,  
587 2013, 2012) In common carp, GnIH-3 (GnIH-III) decreases *fshβ* mRNA expression at  
588 100 μM, but not at 10 μM (Peng et al., 2016). In *Astyanax altiparanae* males, zebrafish  
589 GnIH-3 attenuates the cGnRH2 stimulatory action on *fshβ* mRNA expression by pituitary  
590 explants, without any effect on basal *fshβ* expression level (Branco et al., 2019). In cultured  
591 pituitary cells of grass puffer, it has been reported a stimulatory effect of gfLPXRFa-1 on *fshβ*  
592 mRNA levels (Shahjahan et al., 2011). Finally, in chicken, GnIH depresses *fshβ* mRNAs  
593 (Ciccione et al., 2004).

594 In a representative of the most ancient lineage of vertebrates, the lamprey,  
595 intraperitoneal injections of GnIH (LPXRFa-2) stimulates gonadotropin β expression (Osugi  
596 et al., 2012).

597 In the European eel, we have shown an inhibitory effect of all three aaGnIH peptides  
598 on *gpα* expression *in vitro*. Such an effect was also reported *in vitro* in chicken (Ciccione et  
599 al., 2004), as well as in some teleosts [zebrafish (Spicer et al., 2017); tongue sole (Wang et al.,  
600 2019b)].

601 We have also reported an inhibitory effect of eel GnIH peptides on the expression of  
602 *gnrh-r2* expression *in vitro*, as recently shown with kisspeptin (Pasquier et al., 2018) and  
603 tachykinin (Campo et al., 2018) peptides. In the sea bass pituitary, GnRHR-II-1a is the main  
604 GnRH receptor and co-localizes with all LH- and some FSH-producing cells (González-  
605 Martínez et al., 2004; Moncaut et al., 2005). Consistently, it has been shown that  
606 intracerebroventricular (icv) injection of sbGnIH-2 significantly reduces the expression of  
607 pituitary *gnrhr-II-1a* (Paullada-Salmerón et al., 2016b). In contrast, the second sea bass GnIH  
608 peptide, sbGnIH-1, does not induce any changes in pituitary *gnrhr-II-1a* expression  
609 (Paullada-Salmerón et al., 2016b). In the sea bass brain, the main GnRH receptor is GnRHR-  
610 II-2b and its expression is not affected by icv injection of sbGnIH-1 or sbGnIH-2 (Paullada-  
611 Salmerón et al., 2016b). Our study in the European eel is the first one in vertebrates showing  
612 a direct pituitary inhibitory effect of GnIH on GnRH receptor expression.

613 While in the eel, GnIH has an inhibitory effect on gonadotropin expression *in vitro*, no  
614 effect has been demonstrated on *gh* mRNA levels. However, a predominant stimulatory effect  
615 of GnIH is observed in other teleosts *in vitro* [goldfish (Moussavi et al., 2014); grass puffer  
616 (Shahjahan et al., 2016); tongue sole (Wang et al., 2019b)] except, in the European sea bass,  
617 in which *in vivo* experiment have shown that icv injection of sbGnIH-2 (but not sbGnIH-1)  
618 induces a decrease in *gh* mRNA levels (Paullada-Salmerón et al., 2016b). To the best of our  
619 knowledge, only one study on *gh* expression is available in mammals. In ovines, RFRP-3 has  
620 no effect *in vitro* on the expression of *gh* [primary culture of sheep pituitary cells: (Sari et al.,  
621 2009)].

622 In conclusion regarding the neuroendocrine effect of GnIH in the eel, all the 3 peptides  
623 encoded by the eel *gnih* gene are able to exert an inhibitory action on the expression of  
624 pituitary genes involved in the pituitary control of reproduction (*lh $\beta$* , *fsh $\beta$* , *gp $\alpha$*  and *gnrh-r2*).  
625 These results demonstrate that GnIH peptides may exert a dual inhibitory action on the  
626 gonadotropic function in the eel: they inhibit the expression of gonadotropin subunits (*lh $\beta$* ,  
627 *fsh $\beta$* , *gp $\alpha$*  subunits) and also may reduce the responsiveness of gonadotropic cells to GnRH  
628 via the inhibition of the expression of GnRH receptors. In addition to dopamine (Jolly et al.,  
629 2016; Vidal et al., 2004) and some other neuropeptides such as kisspeptins (Pasquier et al.,  
630 2018) and tachykinins (Campo et al., 2018), GnIH may thus represent one of the brain actors  
631 involved in the prepubertal blockade of eel sexual maturation. This inhibition is specific, as  
632 we have observed no effect on the expression of other pituitary hormones, *gh* and *tsh $\beta$* . The  
633 common inhibitory effect of the 3 eel GnIH peptides maybe related to the relative similarity  
634 of their C-terminal coil 3D structure and sequences (LPLRFamide, LTTRFamide and  
635 LPQRFamide). This is the first teleost species in which all three existing peptides have a  
636 similar inhibitory effect on pituitary hormone expression *in vitro*. Further studies should also  
637 investigate their possible synergistic effects. Our results in a basal teleost are in agreement  
638 with previous data demonstrating an inhibitory role in reproduction of GnIH in ancestral  
639 vertebrates, which would have been conserved in mammals, birds and most teleosts, whereas  
640 a change to a stimulatory role would have occurred in some teleost and agnathans (Tsutsui et  
641 al., 2018). Our finding of the inhibitory effect of GnIH in the eel opens new research avenues  
642 to investigate the *in vivo* effects of GnIH peptides and GnIH receptor antagonists in this  
643 species of basic and applied relevances.

644

#### 645 **4.2. Evolutionary scenario of GnIH in fish**

646 Previous studies have inferred that vertebrate *gnih* (*npvf*) originates from an ancestral

647 PQRF-encoding gene present at least in a chordate ancestor. One of the two rounds of whole  
648 genome duplications (1R/2R) that occurred early in the vertebrate lineage would have given  
649 rise to *gnih* and its sister gene *npff* (for review: Tsutsui et al., 2018). Up to four *gnih*  
650 (*npvf*)/*npff* paralogs could have thus been expected in vertebrates from the 1R and 2R, while  
651 only two paralogs (a single *gnih* and a single *npff*) are retrieved in agnathans (sea lamprey) as  
652 well as in basal sarcopterygians (coelacanth) and basal actinopterygians (spotted gar). This  
653 suggests an early loss of two *gnih* (*npvf*)/*npff* paralogs shortly after 1R/2R.

654 In the present study, we have identified a *npff* gene but did not retrieve any *gnih* gene  
655 in the recently released chondrichytan genomes. This suggests the loss of *gnih* in this lineage,  
656 which deserves further investigations.

657 Previous studies in various teleosts reported a single *gnih* gene. Considering that a  
658 third round of whole genome duplication (3R) specifically occurred at the basis of the teleost  
659 lineage, two *gnih* paralogs could have been expected. We addressed this question in the  
660 present study, with a special focus on basal teleosts.

661 Recently, new genome assemblies have been released including representative species  
662 of the most basal groups of teleosts, elopomorphs (*Anguilla* species), osteoglossomorphs (*e.g.*  
663 arowana and arapaima), and of relatively basal groups among clupeocephala, clupeiforms  
664 (herring, *Clupea harangus*, and pilchard, *Sardina pilchardus*), gymnotiforms (electric eel,  
665 *Electrophorus electricus*). Previous investigations on the evolution of various genes have  
666 shown that teleost basal species have often retained more paralogous genes, whatever their  
667 origin (from 1R/2R or 3R), than more recently emerged teleosts (*e.g.* kisspeptin receptors:  
668 Pasquier et al., 2012; leptin receptors: (Morini et al., 2015); progesterone receptors: (Morini et  
669 al., 2017)).

670 Taking advantage of the new genomes available we have traced the evolutionary  
671 scenario of *gnih* (*npvf*) in teleosts. Our search in genomic databases has revealed only a single

672 *gnih* gene in representative species of the most basal groups of teleosts, elopomorphs and  
673 osteoglossomorphs, as well as in representatives of basal clupeocephala group, clupeiforms,  
674 and of more recent groups of clupeocephala. This suggests the conservation of only a single  
675 3R-duplicated *gnih* paralog in all extant teleosts. The synteny analysis indicated the orthology  
676 of the single conserved *gnih* paralog among elopomorphs, osteoglossomorphs and  
677 clupeocephala. This allows us to infer that the loss of one of the two 3R-duplicated *gnih*  
678 paralogs occurred shortly after the 3R, before the emergence of the most basal groups  
679 elopomorphs and osteoglossomorphs, leading to the presence of a single *gnih* in the whole  
680 teleost lineage. Accordingly, the possible variations in *gnih* physiological functions among  
681 teleosts cannot be related to the conservation of one *versus* the other of the 3R-paralogs, since  
682 the same single *gnih* 3R-paralog has been conserved in all teleosts.

683         We retrieved two *gnih* genes in salmonids, such as Atlantic salmon and rainbow trout,  
684 but the phylogeny analysis showed that the duplicated genes clustered in a same clade with  
685 the pike *gnih* branching at the basal position. Salmonids are known to have undergone a 4th  
686 genomic duplication (salmonid 4R). The presence of two paralogs in salmonids while a single  
687 gene was found in pike, member of the esoxiforme group that have diverged from salmonids  
688 before the 4R suggests that the salmonid duplicated *gnih* derived from the 4R.

689

690         In conclusion, we identified in the eel a single *gnih* gene, expressed in the  
691 diencephalon and encoding three putative GnIH peptides. The three eel GnIH peptides were  
692 synthesized and shown to inhibit the expression of gonadotropin subunits and GnRH receptor  
693 by eel pituitary cells. This inhibitory action of GnIH in a basal teleost is in agreement with the  
694 hypothesis of an ancestral inhibitory role of GnIH in the neuroendocrine control of  
695 reproduction in vertebrates. Phylogeny and synteny analyses supported an early loss of one of

696 the 3R-paralog of GnIH leading to the presence of a single GnIH in the teleost lineage as in  
697 other vertebrates.

698

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706

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1034

1035 **Figure Legends**

1036 **Figure 1:** Consensus tree of vertebrate GnIH (NPVF) preproprotein sequences.

1037 The tree was inferred by Maximum Likelihood using PhyML and rooted using the lamprey  
1038 (*P. marinus*) LPXRF and amphioxus (*B. japonicum*) PQRFa precursor sequences. Bootstrap  
1039 values from 100 replicates are indicated for each node. The species name is followed by the  
1040 chromosome or scaffold number carrying the GnIH. The sequence references are given in the  
1041 Supplementary Table 1.

1042

1043 **Figure 2:** Conserved synteny of the *gnih* (*npvf*) gene in actinopterygians and impact of the  
1044 teleost-specific whole genome duplication.

1045 The spotted gar (*L. oculatus*) *gnih* genomic region is used as a reference. This region is  
1046 duplicated in teleosts after teleost-specific whole genome duplication (TWGD or 3R). The  
1047 duplicated genomic regions of Japanese eel (*A. japonica*), European eel (*A. anguilla*),  
1048 arowana (*S. formosus*), zebrafish (*D. rerio*), pike (*E. lucius*) and medaka (*O. latipes*) are  
1049 illustrated. *Gnih* genes are in blue. 3R *gnih* paralogous genes, which have been lost, are in  
1050 grey with a red cross. Gene positions are given in Mega base below the genes. Full names and  
1051 references of *gnih* and neighbouring genes are given in the Supplementary Table 3.

1052

1053 **Figure 3:** Tissue distribution of eel *gnih* transcripts.

1054 *Gnih* mRNA levels were analyzed by qPCR in various parts of the brain, in the pituitary and  
1055 peripheral tissues in silver female European eels. Olfactory bulbs (OB), telencephalon (Tel),  
1056 diencephalon (Di), mesencephalon (Mes), *cerebellum* (Cer), *medulla oblongata* (MO),  
1057 pituitary (Pit), eye, gill, liver, kidney, intestine, spleen, muscle, adipose tissue (Fat), and

1058 ovary. The relative abundance of *gnih* mRNA was normalised to the amount of total RNA.  
1059 Each bar represents mean  $\pm$  SEM from 8 individuals.

1060

1061 **Figure 4:** Predicted three-dimensional structures of eel GnIH peptides.

1062 The three-dimensional (3D) structures of European eel GnIH-1, -2 and -3 peptides (aaGnIH-1,  
1063 aaGnIH-2, aaGnIH-3) were predicted using I-TASSER server. Models with a C-score  
1064 between 2 and -5 are presented. They are visualised rainbow-colored, with the N-terminal  
1065 end in blue and the C-terminal end in red, showing the central part of the peptides in green.

1066

1067 **Figure 5:** Dose-dependent effects of eel GnIH peptides on *lh $\beta$*  expression by primary cultures  
1068 of eel pituitary cells.

1069 Pituitary cells from silver female European eels were treated with various concentrations of  
1070 the three European eel GnIH peptides (aaGnIH-1, aaGnIH-2, aaGnIH-3) for 10 days. mRNA  
1071 levels of luteinising hormone  $\beta$  subunit (*lh $\beta$* ) (A) and reference gene  *$\beta$ -actin* (B) were  
1072 quantified by qPCR. Data of *lh $\beta$*  were normalized against  *$\beta$ -actin*. Each point represents mean  
1073  $\pm$  SEM of three (GnIH-2) or six (GnIH-1 and -3) independent cell culture experiments with  
1074 five well replicates/group/experiment. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$  versus controls, ANOVA.

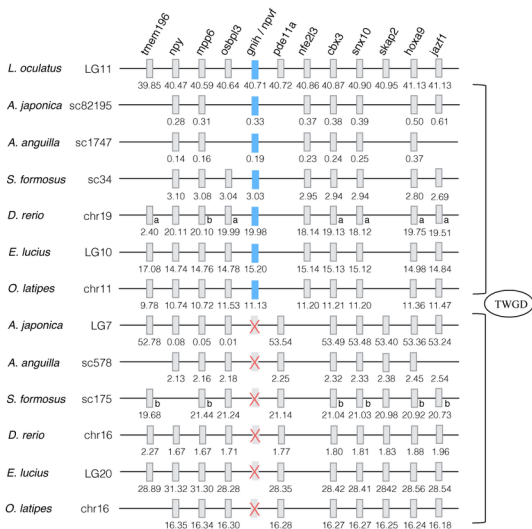
1075

1076 **Figure 6:** Effects of eel GnIH peptides on the expression of various pituitary hormones and  
1077 receptor by primary cultures of eel pituitary cells.

1078 Pituitary cells from silver female European eels were treated with  $10^{-6}$  M of the three  
1079 European eel GnIH peptides (aaGnIH-1, aaGnIH-2, aaGnIH-3) for 10 days. The mRNA levels  
1080 for luteinising hormone  $\beta$  subunit *lh $\beta$*  (A), follicle stimulating hormone  $\beta$  subunit *fsh $\beta$*  (B),  
1081 thyrotropin subunit  $\beta$  subunit *tsh $\beta$*  (C), common glycoprotein hormone  $\alpha$  subunit *gp $\alpha$*  (D),  
1082 growth hormone *gh* (E) and GnRH receptor *gnrh-r2* (F) were quantified by qPCR. Data were

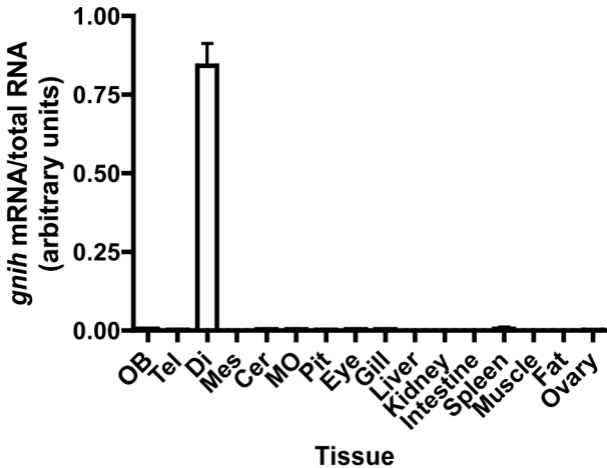
1083 normalized against  $\beta$ -actin. Each bar represents mean  $\pm$  SEM of three (GnIH-2) or six (GnIH-  
1084 1 and -3) independent cell culture experiments with five well replicates/group/experiment. \*,  
1085 P < 0.05; \*\*\*, P<0.001 *versus* controls, ANOVA.

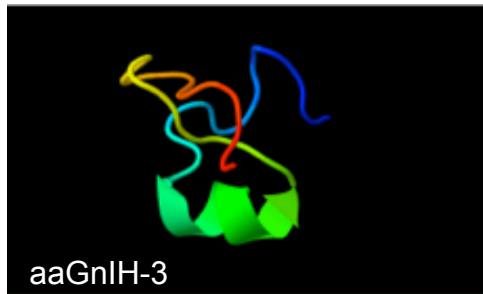
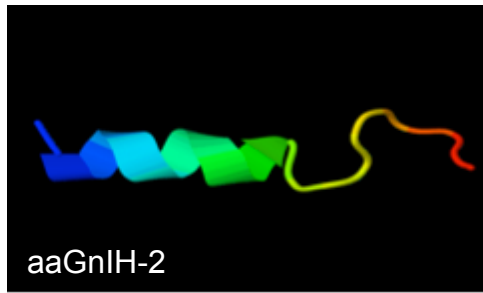
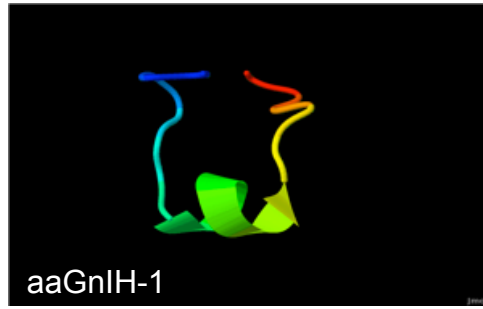




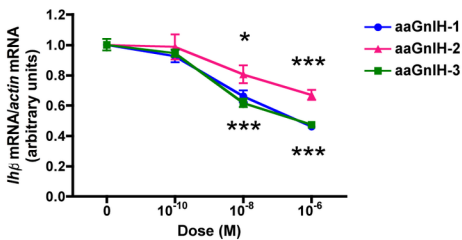
TWGD



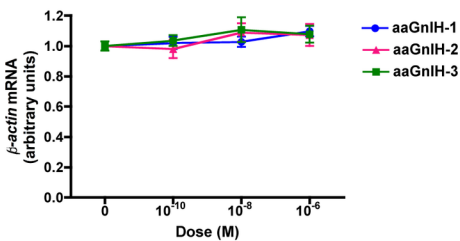




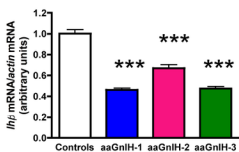
A) *Ihβ*



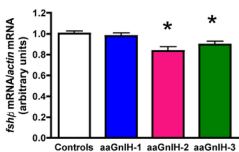
B)  $\beta$ -actin



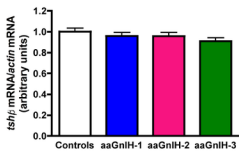
A) *lhβ*



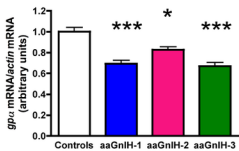
B) *fshβ*



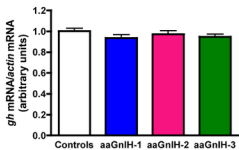
C) *tshβ*



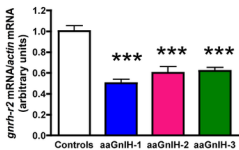
D) *gpa*



E) *gh*



F) *gnrh-r2*



**Table 1:** Comparison of predicted sequences of GnIH peptides in *Anguilla* species.

Aa : *Anguilla anguilla*; ar: *Anguilla rostrata*; aj: *Anguilla japonica*. Amino-acids differing from *A. anguilla* are highlighted in red. For GnIH-2 of *A. japonica*, the proline in position 3 was substituted by a serine in the cloned cDNA (BAV18007.1) but not in the genomic sequence (scaffold BEWY01082450 of the Ajp\_01 assembly).

Peptide	Sequence	Length (aa)
aaGnIH-1	LYPPVAKPALLHAN <b>LPLRF</b> -NH <sub>2</sub>	19
arGnIH-1	LYPPVAKPALLHAN <b>LPLRF</b> -NH <sub>2</sub>	
ajGnIH-1	LYPPVAKPALLHAN <b>LPLRF</b> -NH <sub>2</sub>	
aaGnIH-2	SS P RAATRMLQFPL <b>SLTRRF</b> -NH <sub>2</sub>	20
arGnIH-2	SS P RAATRMLQFPL <b>SLTRRF</b> -NH <sub>2</sub>	
ajGnIH-2	SS P/ <b>S</b> RAA <b>AQ</b> MLQFPL <b>SLTRRF</b> -NH <sub>2</sub>	
aaGnIH-3	SPETDSPIALPCHQCARMGGVASPSAT <b>LPQRF</b> -NH <sub>2</sub>	32
arGnIH-3	SPETDSPIALPCHQCARMGGVASPSAT <b>LPQRF</b> -NH <sub>2</sub>	
ajGnIH-3	SPETDSPIALPCHQCAR <b>I</b> GGVASPSAT <b>LPQRF</b> -NH <sub>2</sub>	