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Tachykinin-3 Genes and Peptides Characterized in a Basal Teleost, the European Eel: Evolutionary Perspective and Pituitary Role

Aurora Campo1, Anne-Gaëlle Lafont1, Benjamin Lefranc2, Jérôme Leprince2, Hervé Tostivint3, Nédia Kamech1, Sylvie Dufour1 and Karine Rousseau*

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In mammals, neurokinin B (NKB) is a short peptide encoded by the gene tac3. It is involved in the brain control of reproduction by stimulating gonadotropin-releasing hormone (GnRH) neurons, mainly via kisspeptin. We investigated tac3 genes and peptides in a basal teleost, the European eel, which shows an atypical blockade of the sexual maturation at a prepubertal stage. Two tac3 paralogous genes (tac3a and tac3b) were identified in the eel genome, each encoding two peptides (NKBa or b and NKB-related peptide NKB-RPa or b). Amino acid sequence of eel NKBa is identical to human NKB, and the three others are novel peptide sequences. The four eel peptides present the characteristic C-terminal tachykinin sequence, as well as a similar alpha helix 3D structure. Tac3 genes were identified in silico in 52 species of vertebrates, and a phylogeny analysis was performed on the predicted TAC3 pre-pro-peptide sequences. A synteny analysis was also done to further assess the evolutionary history of tac3 genes. Duplicated tac3 genes in teleosts likely result from the teleost-specific whole genome duplication (3R). Among teleosts, TAC3b precursor sequences are more divergent than TAC3a, and a loss of tac3b gene would have even occurred in some teleost lineages. NKB-RP peptide, encoded beside NKB by tac3 gene in actinopterygians and basal sarcopterygians, would have been lost in ancestral amniotes. Tissue distribution of eel tac3a and tac3b mRNAs showed major expression of both transcripts in the brain especially in the diencephalon, as analyzed by specific qPCRs. Human NKB has been tested in vitro on primary culture of eel pituitary cells. Human NKB dose-dependently inhibited the expression of lhβ, while having no effect on other glycoprotein hormone subunits (fshβ, tshβ, and gpα) nor on gh. Human NKB also dose-dependently inhibited the expression of GnRH receptor (gnrh-r2). The four eel peptides have been synthesized and also tested in vitro. They all inhibited the expression of both lhβ and of gnrh-r2. This reveals a potential dual inhibitory role of the four peptides encoded by the two tac3 genes in eel reproduction, exerted at the pituitary level on both luteinizing hormone and GnRH receptor.

Keywords: tachykinin-3, neurokinin B, phylogeny, synteny, pituitary cell culture, luteinizing hormone, GnRH-R, teleost
INTRODUCTION

Tachykinins are peptides mainly produced by brain and gut in mammals [for reviews see Ref. (1, 2)]. The most known tachykinin peptides are neurokinin A (NKA), substance P (SP), and neurokinin B (NKB). While NKA and SP are encoded by the tac1 gene (also named preprotachykinin A gene, PPT-A, or PPT-1), NKB is coded by the tac3 gene (also named PPT-B or PPT-II, and tac2 in rodents) [for reviews see Ref. (2–4)]. A second peptide encoded by the tac3 gene has been recently found in teleosts and was either named neurokinin F (NKF) [zebrafish, Danio rerio (5)] or NKB-related peptide (NKB-RP) [grass carp, Ctenopharyngodon idella (6); tilapia, Oreochromis niloticus (7)]. A tac4 gene (also named PPT-C or PPT-III) encodes other tachykinins in mammals: hemokinin-1 and endokinins [for reviews see Ref. (3, 4, 8)]. An evolutionary scenario is that an ancestral gene has given rise to four tac genes after the two whole genome duplication rounds (1R/2R) in early vertebrates (12, 13), reversible in adulthood (14) Similarly, although fertile, tac3 or tac5 null mice exhibited central reproductive defects such as an abnormal estrous cyclicity (15, 16). In addition, in vivo studies in different mammals have shown the stimulatory effect of NKB on gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) secretion mainly via stimulation of the kisspeptin system [mice (17, 18); sheep (19); monkey (20); men and women (21, 22); and rat (23)] [for review see Ref. (24)]. Stimulatory effect of TAC3 on follicle-stimulating hormone (FSH) secretion has also been reported [mice (17, 18); dog (25); monkey (26); and men (22)]. However, a lack of effect or inhibitory action on gonadotropins has also been documented [for reviews see Ref. (27, 28)]. In mammals, NKB is coexpressed with kisspeptin (Kiss) and dynorphin in neurons of the arcuate nucleus of the hypothalamus, which are therefore called KNDy neurons [for review see Ref. (29)]. KNDy neurons project to GnRH neurons and regulate their activity [for reviews see Ref. (24, 27, 28)]. Ablation of KNDy neurons in female rats resulted in hypogonadotropic hypogonadism (30).

In some teleosts, two tac3 genes have been identified, likely resulting from the teleost-specific whole genome duplication (3R) (10). The role of TAC3 on gonadotropic axis has been studied in some teleosts both in vivo and in vitro. In vivo data showed increase in gonadotropin expression and release after treatment with NKB peptides [zebrafish (5); tilapia (31); goldfish, Carassius auratus (32); and orange-spotted grouper, Epinephelus coioides (33)]. However, a recent study has also reported the absence of effect of NKB on gonadotropins [tilapia (7)]. In vitro, NKB peptides have been shown to be either stimulatory [tilapia (31); striped bass, Morone saxatilis (34)], inhibitory [tilapia (7)] or without effect on gonadotropins [grass carp (6); tilapia (7); and orange-spotted grouper (33)]. In contrast to mammals, tachykinin-3 is not coexpressed with kisspeptin in teleost brain [zebrafish (35); striped bass (34)]. However, TAC3 peptides could downregulate kiss2 expression in striped bass [both NKB and NKB-RP in vitro and in vivo (34)] and in tilapia [NKB-RP only, in vivo (7)], suggesting that tachykinin peptides may act indirectly on GnRH via the kisspeptin system, like in some mammals (24, 27). In addition, colocalization of tac3 in lhβ cells in tilapia pituitary (31) points out that TAC3 peptides as possible direct modulators of gonadotropin secretion in teleosts.

In the European eel, Anguilla anguilla, the blockade of the puberty is related to a low stimulation by GnRH (36) and a strong inhibitory control by dopamine (37) [for review see Ref. (38)]. We aimed at investigating TAC3 potential involvement in the control of gonadotropic axis in the European eel. In addition, studies of the tachykinin genes in the European eel, a member of an early group of teleosts (elopomorphs), may provide new insights on ancestral regulations.

In our study, we identified tac3 genes and encoded peptides in the European eel by in silico data mining and cloning. Phylogeny and synteny analyses were performed to infer the molecular evolution of the TAC3 peptides throughout teleost radiation. Tissue distribution of the two eel tac3 gene expression was investigated by specific qPCRs. The four predicted eel TAC3 peptides were synthesized and tested for their in vitro effect on pituitary hormone and receptor expressions by eel pituitary cells.

MATERIALS AND METHODS

Animals

European female eels (A. anguilla) were at the prepubertal “silver” stage, corresponding to the end of the continental stage of the eel life cycle, previous to migration to the ocean for reproduction. They were purchased from Gebr. Dil import-export BV (Akersloot, The Netherlands) and transferred to MNHN, France. Animals were anesthetized by cold and then killed by decapitation under the supervision of authorized person (KR; No. R-75UPMC-F1-08) according to the protocol approved by Cuvier Ethic Committee France (No. 68–027).

In Silico Prediction of tac3 Genes

Tac3 sequences from vertebrate species were retrieved from the Ensembl release 91 and NCBI databases. Additional blasts were performed using TBLASTN algorithm of the CLC Main Workbench 6 software (QIAGEN Bioinformatics) in the teleost genomes and multiorgan transcriptomes downloaded from NCBI, Ensembl and Phylofish (39). The sequences of zebrafish tac3a (Gene ID: 100320820) and tac3b (Gene ID: 569642) (5) were translated with the EXPASY online tool (40) and used as queries. The obtained predicted sequences of the tachykinin pre-pro-peptide were added to the query list for a new multiblast in the next species, up to 52 species in total.

For the European eel, blast analyses were performed on both available draft genomes [Illumina (40) and nanopore (41)] and on
Cloning and Sequencing of Eel tac3 cDNAs
The anterior part of the brain including olfactory bulbs (OBs), telencephalon, and di-/mesencephalon was dissected and stored in RNA later (Ambion Inc., Austin, TX, USA) at 4°C (24 h), then at −20°C until extraction. Total RNA was extracted using mechanical homogenization in Trizol Reagent (Invitrogen, Cergy-Pontoise, France), according to the manufacturer’s instructions. Samples were homogenized by TissueLyser II (QIAGEN, Hilden, Germany) and further treated with deoxyribonuclease I (Roche, Meylan, France). RNA quantifications have been performed using a nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and purchased from Eurofins (Hamburg, Germany). The PCR reactions were carried out on a MyCycler Thermal Cycler (Bio-Rad, Marne-la-Coquette, France) using the GoTaq PCR Core System I (Promega, Charbonnières, France) under the following conditions: 3 min at 94°C, then 35 cycles of 1 min at 94°C, 1 min 2°C under the Tm of the oligonucleotide with the lowest Tm, and 1 min at 72°C; 7 min at 72°C. The amplification products were subcloned into the pGEM-T Easy vector (Promega) and sequenced (Value Read Sequencing at MGW Biotech, Ebersberg, Germany).

Phylogeny Analysis
18 Sarcopterygian, 1 chondrichthyan, and 59 actinopterygian TAC3 pre-pro-peptide amino acid sequences were aligned using MUSCLE included in SeaView v. 4.6.1 (43). Alignment was mainly adjusted for optimization of key regions such as cleavage sites and signal peptide.

Phylogenetic analysis of the TAC3 alignment was achieved using a maximum likelihood method, RAxML black-box7 (44), with 1,000 bootstrap replicates and JTT substitution matrix. The TAC3 pre-pro-peptide sequence from the elasmobranchii elephant shark (Callorhinus milii) was chosen as outgroup. The resulting phylogenetic tree was displayed using Figtree v1.4.3. Nodes were collapsed for bootstrapping values below 50% using Mesquite.

Cloning and Sequencing of Eel tac3 cDNAs
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was performed using the RasWin Molecular Graphics software v. 2.7.5.2.11

**Pituitary Cell Culture**

**Dispersion and Culture**

Dispersion and primary culture of pituitary cells, using 30–40 female eel pituitaries per cell culture experiment, were performed as described in Ref. (49) and as recently used for the test of eel kisspeptins (47). Cultures were performed in serum-free culture medium [M199 with Earle’s salt, sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml fungizone (Gibco, Illkirch, France) at 18°C under 3% CO₂ and saturated humidity].

**Treatments**

Human NKB (Sigma-Aldrich, Saint-Quentin Fallavier, France) and eel TAC3 peptides stock solutions (10⁻⁴ M) were prepared in NaOH 0.1 M and stored at −20°C. Stock solutions were diluted in the culture medium just before the addition to the culture wells. The treatments started at day 0 (24 h after cell plating) and five wells per treatment (62,500 cells/well) were used as replicates. Culture medium was changed and peptide solution renewed every 3 or 4 days (day 4 and day 7), and culture was stopped at day 10. The effects of the treatments were tested on three independent experiments performed on cell cultures from different batches of fish and figures display the results of representative experiments. For human NKB, a range of concentrations from 10⁻¹² to 10⁻⁴ M was tested according to previous *in vitro* studies with neuropeptides (47, 50). For synthetized eel NKB and NKB-RP peptides, 10⁻⁷ M was chosen as submaximal response could be observed with 10⁻⁶ M human NKB.

**RNA Extraction and cDNA Synthesis**

Total RNA was directly extracted as previously described (47, 50). Briefly, cells were washed with sterile PBS (Gibco) and lysed with Cell-to-cDNA™ II Cell Lysis II Buffer (Ambion; 80 µl/well). The lysates were digested with RNase-free DNase I (Roche). Eight microliters of RNA solution of each sample were then reverse-transcribed with a SuperScript III First Strand cDNA Synthesis Kit (Invitrogen) and stored at −80°C. The cDNA samples obtained were stored at −20°C until qPCR.

**Quantitative Real-Time PCR (qPCR) Primers**

Primers for eel *tac3a* and *tac3b* were designed based on sequences of European eel TAC3 pro-peptides using Primer 3 (Table S2 in Supplementary Material) and purchased at Eurofins. Amplicon sizes were 140 bp for *tac3a* and 190 bp for *tac3b*. Forward and reverse primers of each couple were located in different exons to prevent amplification of genomic DNA. To assess the specificity of the qPCR primers, each couple was tested for its inability to amplify the transcript of the other *tac3* gene.

The housekeeping gene was β-actin as previously reported (50, 51). Primers for eel *lhβ*, *fshβ*, β subunit of thyroid-stimulating hormone (*tshβ*), common α subunit of glycoprotein (*gpa*), type 2 GnRH receptor (*gnrh-r2*), and growth hormone (*gh*) have already been described [Table S2 in Supplementary Material (51–53)]. European eel possesses three GnRH receptors (GnRH-R1a, GnRH-R1b, and GnRH-R2) (53), but *gnrh-r1a* and *gnrh-r1b* expression was below the threshold of detection in cultures of pituitary cells (47), and thus were not assayed in this study.

**SYBR Green Assay**

Quantitative PCR assays were performed using the LightCycler® System (Roche) with SYBR Green I sequence-unspecific detection as previously described (47, 50). Briefly, the qPCRs were prepared with 2 µl of RNase-free water (Ambion), 2 µl of SYBR Green master mix (Roche), 1 µl of each forward and reverse primer (500 nM final concentration), and 4 µl of diluted cDNA template. The protocol was an initial step of polymerase activation for 10 min at 95°C; then 41 cycles (β-actin, *gh*, *gpa*, *lhβ*, *fshβ*, and *tshβ*) or 45 cycles (*tac3a* and *tac3b*) of 10 s at 95°C for denaturing, 5 s at 60°C for annealing, 10 s at 72°C for primer extension and a single final extension step of 5 min at 72°C. For *gnrh-r2*, the protocol was an initial step of polymerase activation for 10 min at 95°C; 42 cycles of 10 s at 95°C, 7 s at 61°C, 4 s at 72°C and a single final extension step of 5 min at 72°C. Each program ended with a melting curve analysis by slowly increasing the temperature (0.01°C/s) from 68 to 95°C with a continuous registration of changes in fluorescence intensity. Serial dilutions of cDNA pool of brain (tissue distribution) or pituitary cells (cell culture) were used as a standard curve. One chosen dilution was also included in each run as a calibrator. Normalization of data was performed using total RNA levels (tissue distribution) and β-actin mRNA level (cell culture experiments).

**Statistical Analysis**

Data are presented as the mean ± SEM. Mean values were compared by Student’s *t*-test or one-way ANOVA followed by Tukey’s multiple comparison test, using Instat (GraphPad Software Inc., San Diego, CA, USA). Differences between groups with *P* < 0.05 were considered statistically significant.

**RESULTS**

**Characterization of European Eel *tac3* Genes, Transcripts, and Peptides**

**In Silico Identification of Eel *tac3* Genes and Cloning of Transcripts**

Two *tac3* genes were identified in the European eel genome as well as in the transcriptome (Figure 1). Using European eel specific *tac3a* primers designed on eel *tac3a* predicted genomic

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**TABLE 1 | Sequences of European eel (*Anguilla anguilla*) predicted TAC3 peptides.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Peptide name</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>tac3a</td>
<td>NKBa</td>
<td>DMHDFVGLM-NH₂</td>
</tr>
<tr>
<td></td>
<td>NKB-RPa</td>
<td>YNGIDYDSPVGLM-NH₂</td>
</tr>
<tr>
<td>tac3b</td>
<td>NKBb</td>
<td>DMDDIFVGLM-NH₂</td>
</tr>
<tr>
<td></td>
<td>NKB-RPb</td>
<td>YNDOYDTFVGLM-NH₂</td>
</tr>
</tbody>
</table>

The predicted sequences are the same for Anguilla japonica and Anguilla rostrata.
Fig. Ure 1 | European eel tac3a and tac3b sequences. Nucleotide and deduced amino acid sequences of tac3a (a) and tac3b (b). Nucleotides (top) are numbered from 5′ to 3′. The amino acid residues (bottom) are numbered beginning with the first methionine residue in the ORF. The asterisk (*) indicates the stop codon. The seven exons are indicated. The amino acids of the signal peptide are underlined. The TAC3 peptides encoded by tac3a are in blue and by tac3b in red. Related peptides are in italic. The cleavage sites are marked by a square and amidation sites by a circle.

**Phylogeny Analysis of TAC3**

Based on an alignment of 78 TAC3 pre-pro-peptidic amino acid sequences (Figure S1 in Supplementary Material), and assuming the elephant shark *C. milii* sequence as outgroup, a phylogenetic tree was generated using the maximum likelihood method (the list of sequences and accession numbers is provided in Table S1 in Supplementary Material) (Figure 2).

As shown in Figure 2, the TAC3 pre-propeptide sequences are clustered into two main clades: sarcopterygians and actinopterygians. In the sarcopterygian clade, TAC3 sequences of birds have diverged, as indicated by the long branch of this group. The spotted gar TAC3 sequence branches at the base of the actinopterygian clade. In most teleosts, two TAC3 (a and b) were found, TAC3a being present in all investigated species, while TAC3b not being retrieved in a few species even with available genome such as a clupeomorph (herring), and an acanthopterygii (medaka). Teleost TAC3b sequences form a single clade, including elopomorph (eel) and osteoglossomorph (arowana) sequences branching at its basis. By contrast, a well-supported clade is observed

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**Sequence and Primer Design**

sequence, and tac3b primers designed on eel tac3b predicted genomic sequence (Table S2 in Supplementary Material), PCRs were performed on brain cDNAs. A CDS of 378 bp was characterized for each tac3 (tac3a: MH107060; Figure 1A and tac3b: MH107060; Figure 1B). Once translated, both pre-pro-peptide sequences were 125 aa long. BLASTN analyses performed on the European eel draft genome, using the present tac3a and tac3b cloned sequences as queries, revealed that each transcript is encoded by 7 exons. The pre-pro-peptide is encoded between exons 2 and 7 and the mature peptides by exon 3 (NKB-RP) and exon 5 (NKB) (Figure 1).

**Prediction of Mature European Eel TAC3 Peptides**

Both eel pre-pro-peptide sequences encode two peptides (Figure 1). These peptides were delimited by cleavage sequences at both ends (KR at N-terminal and RR at C-terminal). The C-terminal end of the sequence shows a glycine before the cleavage site, which indicates an amidation site.

The eel NKBa peptide (10 aa) encoded by tac3a gene (Figure 1; Table 1) has the same sequence as human NKB. This sequence is also conserved in various sarcopterygians (coelacanths, sauropods, and mammals) as well as in the non-teleost actinopterygian, spotted gar (Figure S1 in Supplementary Material). By contrast, variations in the sequence are observed in all other teleosts studied (Figure S1 in Supplementary Material). The eel NKBb (10 aa) peptide encoded by tac3a gene as well as both peptides, eel NKBb (10 aa) and eel NKB-RPb (13 aa), encoded by tac3b gene (Figure 1; Table 1) are novel peptide sequences. These peptides have the same sequences in the three eel species studied (European, American, and Japanese eels) (Table 1; Figure S1 in Supplementary Material).
only for clupeocephala TAC3a sequences. Basal teleost TAC3a sequences are not included in this clade: eel TAC3a sequences branch at the basis of all teleost TAC3a and TAC3b sequences, and arowana TAC3a is in polytomy with clupeocephala TAC3a and teleost TAC3b. From this phylogenetic analysis, we could suggest that the two teleost Tac3 genes likely resulted from 3R, but the classification of the basal teleost TAC3 sequences needed to be further assessed by synteny analysis.

**Synteny Analysis of tac3 Genomic Region**

To further resolve the origin and nomenclature of the duplicated eel tac3a and tac3b, we performed a synteny analysis (Figure 3) on the tac3 genomic region of representative species of various teleost superorders: European eel (elopomorph), golden arowana (osteoglossomorph), Atlantic herring (clupeomorph), zebrafish (ostariophysi), medaka, tilapia, stickleback, and fugu (acanthopterygii). The spotted gar, a non-teleost actinopterygian, was chosen as the reference species in this synteny analysis. In all teleosts, the tac3 genomic region was duplicated in agreement with the 3R (Figure 3). For tac3, synteny analysis highlights the loss for tac3b in some species such as herring and medaka. As these two species belong to different teleost superorders/orders, this indicates independent recurrent events of tac3b loss throughout teleost radiation: at least in the clupeomorph/clupeiform lineage and in the acanthopterygii/beloniform lineage. Some tac3 neighboring genes 3R-paralogs were conserved in all studied teleosts: c1galt1 (except in golden arowana) and b4galnt1. By contrast, for other tac3 neighboring genes, only a single 3R-paralog was conserved. For instance, scl26a10 was conserved on tac3a paralogon, but lost on tac3b paralogon, including in eel and arowana. Conversely, 3R-paralogs of stat2, apof, and os9 were conserved on tac3b paralogon and lost on tac3a paralogon, including in eel and arowana. These syntenic data allow us to definitely assign eel and arowana duplicated tac3 genes as tac3a and tac3b, respectively.

To further assess these orthologies, we also performed phylogeny analyses on tac3 neighboring genes, c1galt1 (Figure S3 in Supplementary Material) and b4galnt1 (Figure S5 in Supplementary Material), the 3R-duplicated paralogs of which have been conserved in most teleosts. For c1galt1 (Figure S3 in Supplementary Material), teleosts sequences located on paralogon a did not group into a clade; however, a well-supported clade grouped all teleost sequences located on paralogon b, including those of eel and arowana branching at the basis of this clade. This phylogeny is in agreement with the respective assignment of eel
FIGURE 3 | Syntenic region of tac3 in key teleost species and the actinopterygian spotted gar. Spotted gar tac3 genomic region is used as a reference. This region is duplicated in teleosts. Blue square includes teleost paralogon a, and red square includes teleost paralogon b. Gene color is preserved for homologous genes. The tac3 genes are highlighted by an orange square. Gene losses are marked with a cross. For each species, chromosome or scaffold is indicated. Below each gene, its position is indicated in 10^6 bp. The full gene names, reference, and detailed genomic locations are given in Table S3 in Supplementary Material.
and arowana tac3 sequences to paralogons a and b. For b4galnt1 (Figure S5 in Supplementary Material), all actinopterygian sequences formed a well-supported clade with the single spotted gar sequences branching at its basis. Teleosts 3R-b4galnt1 duplicated paralogs split into two clades, each one encompassing eel and arowana sequences. Each teleost clade corresponded to the 3R-duplicated genes located on the respective paralogs split into two clades, each one encompassing eel and arowana. This phylogeny fully supported the respective assignment of eel and arowana tac3 sequences to paralogons a and b.

**Tissue Distribution of Eel tac3a and tac3b Transcripts**

Both tac3a and tac3b transcripts were mainly expressed in the brain. Figure 4 displays expression of tac3a and tac3b transcripts in different regions of the eel brain. Eel tac3a and tac3b mRNAs were both predominantly expressed in the diencephalon. Tac3a was also highly expressed in the mesencephalon, with weaker levels in OBs, telencephalon and MO. In addition to diencephalon, tac3b was expressed to a lesser extent in the mesencephalon and telencephalon, with weak levels in Cb and MO and undetectable levels in OBs.

Concerning peripheral tissues, tac3a was weakly expressed in the pituitary and ovary, while tac3b was undetectable. Both tac3a and b were weakly expressed in the intestine, and not detectable in the other tissues investigated (liver, spleen, eye, and gills) (data not shown).

**In Vitro Effect of Human NKB and Eel TAC3 Peptides on Pituitary Hormone and gnrh-r2 Expression by Eel Pituitary Cells**

The effects of commercial human NKB and synthesized eel TAC3 peptides were tested over 10 days in eel pituitary cell culture system as previously described for kisspeptins (47).

**Effects of Human NKB on Pituitary Hormone and gnrh-r2 Expression**

Human NKB peptide dose-dependently inhibited lhβ expression. By contrast, this peptide had no significant effect on the expression of the other glycoprotein hormone subunits (fshβ, tshβ, and gpa) nor on gh (Figure 5A). Human NKB peptide also dose-dependently inhibited gnrh-r2 expression (Figure 5A).

**Effects of Eel TAC3 Peptides on lhβ and gnrh-r2 Expression**

All four eel synthesized TAC3 peptides (NKBa, NKBb, NKB-RPa, and NKB-RPb), as tested at 10⁻⁷ M, significantly inhibited lhβ expression (Figure 5B).

A significant inhibitory effect of all four synthesized eel NKB peptides at 10⁻⁷ M was also observed on the expression of gnrh-r2 (Figure 5C).

**Prediction of the Three-Dimensional Peptide Structure of Eel TAC3 Peptides**

Predicted secondary structures of eel TAC3 peptides were obtained using the I-TASSER server. As described above, eel NKBa peptide sequence is the same as human NKB. Human NKB 3D structure was already reported [PDB ID 1p9f (5)]. For all four eel peptides, the 3D structure was characterized by a single α-helix, as for human NKB (Figure 6). Random coil and turn structure appear in the N-terminal of the related peptides NKB-RPa and NKB-RPb (Figure 6).

**DISCUSSION**

**Two tac3 Genes and Four TAC3 Peptides in the Eel**

We show that the European eel, as well as other eel species, possesses two tac3 genes, each of them encoding two peptides. Tac3a and tac3b genes consist of seven exons as human and rat tac3 genes (54). The sequences that encode NKB (a or b) are located in exon 5, as in human and rat (54), while exon 3 contains the
sequence encoding related peptide (NKB-RPa or b), which has been lost in mammals (55).

Among the four eel TAC3 peptides, NKBa has the same amino acid sequence as human NKB. This sequence, which is also identical in various sarcopterygians (coelacanth, some amphibians, sauropsids, and mammals) as well as in a non-teleost actinopterygian, spotted gar, may represent an ancestral NKB sequence largely conserved throughout vertebrate radiation. This sequence still conserved in the eel, a basal teleost (elopomorph) shows variations in the other teleosts, including another basal

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**FIGURE 5** | Effect of human (A) and eel (B) TAC3 peptides on pituitary hormone and gnrh receptor expression by eel pituitary cells. (A) Primary cultures of pituitary cells were treated with various concentrations (10⁻¹², 10⁻¹⁰, 10⁻⁸, and 10⁻⁶ M) of human neurokinin B (NKB) for 10 days. Pituitary hormones and receptor mRNA levels were quantified by qPCR. (B, C) Pituitary cells were treated with 10⁻⁷ M of eel TAC3 peptides, NKBa, NKbB, NKB-RPa, and NKB-RPb for 10 days. *P<0.05 and **P<0.01 versus controls, t-test or ANOVA. Each point represents mean ± SEM from five well replicates.
representative, the arowana (osteoglossomorph) (Figure S1 in Supplementary Material).

The other three eel peptides are totally new peptides: NKBb and the two neurokinin related peptides (NKB-RPa and NKB-RPb). NKB-RP would be present in amphibians but lacking in reptiles, birds and mammals (2, 5, 55), suggesting a loss of NKB-RP in the amniote lineage. In our study, we show that these related peptides were preserved in actinopterygians, in agreement with previous studies in teleosts (5, 7, 10, 31, 32, 34).

Eel TAC3 peptides all showed the characteristic C-terminal signature motif of FxGLM of tachykinin family, where x is a valine (for review see Ref. (3)). This C-terminal sequence is critical for receptor binding and bioactivities (54). However, in some other teleosts, this C-terminal region of tac3b products is more divergent [FVGLL for zebrafish, goldfish, grass carp, and salmon NKBb; LAALL for sea bass NKBb; FIGLM for goldfish and grass carp NKB-RPb; for review see Ref. (2)].

The structural organization of one precursor containing two peptides is conserved within the human TAC1 and TAC4 precursors but differs from that of human TAC3 precursor, where NKB-RP is missing. In Ciona intestinalis, two tachykinin-like peptides are also produced by one tac gene (56), suggesting that the co-existence of two tachykinin peptides within a single precursor is the ancestral organization of TAC precursors (for review see Ref. (10)).

The 3D model structures of all four eel TAC3 peptides shows a α-helix structure, as previously reported for human NKB as well as for zebrafish TAC3 peptides (5). This helical structure was demonstrated for mammalian NKB in the presence of dodecyl phosphocholine (57) and sodium dodecyl sulfate (58) micelles. In mammals, the formation of a helical conformation in the mid region of TAC3 peptide has been shown to be crucial for tachykinin receptor activation (54). This structure would thus provide to eel TAC3 peptides a binding-competent conformation similar to that of human NKB.

**Eel Duplicated tac3 Genes Come From 3R**

Two tac3 genes are present in the eel, as in most other teleost species investigated, while only a single gene is present in the non-teleost actinopterygian, spotted gar, and in sarcopterygian species. These two paralogs in teleosts likely result from the teleost-specific whole genome duplication (3R). Phylogeny analysis showed that teleost pre-pro-TAC3a amino acid sequences are relatively conserved, while pre-pro-TAC3b sequences have largely diverged in some species. A loss of tac3b would have even happened, independently in some teleost lineages, such as in clupeiform (herring) and beloniform (medaka) as shown herein. An independent loss of tac3b could have also occurred in other teleost subgroups, as suggested by Chen et al. (33) for the grouper (pericorm, serranidae).

The TAC3 phylogeny analysis conducted in this study was not informative enough to allow us to unequivocally classify the two eel TAC3 into the teleost TAC3a and TAC3b. The situation was the same for the two TAC3 from arowana, another representative of basal teleosts. Toward this aim, we performed synteny analysis of tac3 genomic regions in representative species of teleosts, and using a non-teleost actinopterygian, spotted gar, as a reference. The synteny analysis supports that the whole genomic region containing tac3 would have been duplicated in teleosts, probably as a result of 3R. Concerning tac3, our syntenic analysis allowed us to clearly classify the two eel, as well as the two arowana, genes. We considered the eel and arowana tac3 located in the same paralogon as scl26a10 as orthologs to teleost tac3a, and the eel and arowana tac3 located in the same paralogon as stat2, apof, os9 as orthologs to teleost tac3b. Phylogeny analyses of duplicated neighboring genes also supported this conclusion. The two eel tac3 were therefore named accordingly, eel tac3a and tac3b. Our synteny data strengthens the hypothesis of the 3R origin of the two tac3 genes in the eel as in most other teleosts, with the loss of tac3b in some teleost species representing lineage specific events.

**Eel tac3a and tac3b Are Mostly Expressed in the Brain**

Both eel tac3 mRNA were mainly expressed in the diencephalon. This brain region is the major neuroendocrine region of the brain in vertebrates. In mammals, it is where the KNDy neurons are localized (for reviews see Ref. (2, 59)). Eel tac3a and b expressions were also observed in other parts of the brain. Apart from the diencephalon, tac3a gene expression was expressed in the OB and mesencephalon, while the tac3b was expressed in telencephalon and mesencephalon.

A wide brain expression was also observed in other teleosts. In zebrafish, tac3a cerebral expression was observed either mainly in “midbrain” (including optic tectum/mesencephalon-diencephalon and hypothalamus) (5, 35) or predominantly in hypothalamus with low levels in telencephalon and optic tectum-thalamus (10). In this species, tac3b mRNA was mainly expressed in “forebrain” (telencephalon) (5, 10, 35) as well as in hypothalamus (10). In goldfish, both tac3a and tac3b mRNA were found in telencephalon, optic tectum-thalamus, Cb and hypothalamus (32). In grass carp, the expression of the single tac3 (tac3a) was
mostly observed in the OB and hypothalamus (6). Using in situ hybridization, a widespread distribution of tac3 expression was reported in the brain of zebrafish (5, 35), goldfish (32), striped bass (34) and orange-spotted grouper (33). In mammals (human, rat and mouse), a large brain distribution of tac3 transcripts has also been reported, the expression not being restricted to KNy neurons [for reviews see Ref. (1, 59)].

In the pituitary, a weak expression of tac3a but no detectable expression of tac3b was observed in the eel. This differential expression was also reported in goldfish (32). In some species, where only tac3a has been identified, a weak expression was found in the pituitary [orange-spotted grouper (33); grass carp (6)]. By contrast, in zebrafish (5, 35), both genes were found to be expressed in the pituitary. Pituitary expression of tac3a is also observed in mammals (54).

In the ovary, weak expression of tac3a (and none for tac3b) was found in the eel, as in goldfish (32) and orange-spotted grouper (33). In zebrafish, contradictory results were obtained with sexually mature fish: while tac3b gene was found to be expressed in the ovary according to some authors (5, 10), it was tac3a gene in another study (35). Mammalian tac3 gene expression is found in reproductive organs such as placenta, uterus, ovary, oviduct, prostate gland, and testis [for review see Ref. (2)].

NKb was reported to be important in normal follicle growth as well as in estradiol preovulatory and progesterone postovulatory rise in women (21). Direct role of NKb on estradiol production has been recently observed in zebrafish primary cultures of follicular cells and in human cell line derived from a granulosa tumor (60).

A weak expression of both tac3a and b was detected in the intestine of the eel. This tissue expressed tac3a (and not tac3b) in zebrafish and goldfish (32), and the single tac3 gene (tac3a) in grass carp (6) and orange-spotted grouper (33). In mammals, while works described the localization of NKb in intestine, no study has yet provided evidence for the existence of tac3 gene at this level [for review see Ref. (1, 61)]. However, NK3 receptor expression is observed in the gastrointestinal tract and NKb has been shown to induce contractile responses [for review see Ref. (54)].

**Eel TAC3 Peptides Exert a Dual Inhibitory Effect on Pituitary Gonadotropic Function**

In this study, we showed that human NKb as well as all four synthetized eel TAC3 peptides (NKb, NKbb, NKb-RPa, and NKb-RPb) were able to regulate hormone and receptor expression by eel pituitary cells in culture. This reveals a direct pituitary effect of TAC3 peptides in the eel.

All tested TAC3 peptides inhibited lhβ expression, without affecting fshβ transcripts. Studies of the in vitro effect of TAC3 peptides in other teleost species concerned only peptides encoded by tac3a gene and named below NKb and NKb-RP/ NKF. They revealed variable effects on gonadotropins. In striped bass, NKb and NKb-RP (NKf) enhanced LH and FSH releases by primary pituitary cultures, and no effect was observed on lhβ and fshβ transcript levels (34). In tilapia, NKb and NKb-RP (NKF) induced LH and FSH release by primary cultures of mature male pituitary cells (31), in agreement with the presence of NKb receptors (tac3ra and tac3rb) on both cell types (31). By contrast, in the same species, NKb-RP could decrease lhβ and fshβ expression by pituitaries of juvenile mixed-sex animals with no effect of NKb (7). In grass carp, Hu and collaborators (6) reported no variation of LH and FSH expression, cell content and release after NKb or NKb-RP treatment of pituitary cells.

Similarly, orange-spotted grouper NKb and NKb-RP had no effect on gonadotropin mRNA levels in cultured pituitary cells (33). As far as one can tell from the literature, our study is the first to investigate the in vitro effect of TAC3 peptides encoded by both tac3a and tac3b genes in teleosts. We show that these peptides are all able to inhibit lhβ expression in the prepubertal female eel. To the best of our knowledge, only one in vitro study has been reported in mammals. Using gonadotroph cell line LhβT2, Mijiddorj and collaborators observed no effect of NKb on lhβ and fshβ mRNA expression, albeit NKb receptor was detected (62).

When administered in vivo in teleosts, NKb peptides could either increase [zebrafish (5); tilapia (31); and goldfish (32); NKb in orange-spotted grouper (33)] or had no effect [tilapia (7); NKb-RP in orange-spotted grouper (33)] on gonadotropin expression and/or release. Comparing various tachykinins in vivo, Sahu and Kalra (63) were the first to report that NKb-containing implants, in the third ventricle of ovariecotomized rat brain, did not induce any change in LH release. Later, this absence of NKb effect on LH was also shown after either intraperitoneal or intracerebroventricular administration to male mice (64). In this last study, NKb was even ineffective in stimulating GnRH secretion by hypothalamic rat explants (64). However, evidence for stimulatory [mouse (17, 18); monkey (20, 26, 65); sheep (19, 26, 66–68); and human (21, 22)] or inhibitory [rat (69); mouse (70); and goat (71)] effects of NKb on LH have also been documented. Concerning FSH, either stimulatory [mouse (17, 18); dog (25); monkey (26); and man (22)] or no effect [mouse (64); women (21)] of NKb has been reported. These discrepancies in mammals as in teleosts could be due to species, physiological status, or mode of peptide administration.

The downregulation of gnrh-r2 expression that we observed in our study after treatment of eel pituitary cells by commercial human NKb and synthesized eel TAC3 peptides has never been reported before. We have also recently demonstrated a decrease of gnrh-r2 expression after kisspeptin treatment by eel pituitary cells (47), in parallel to a decrease of lhβ expression. These results suggest that a double inhibitory control could be exerted by different neuropeptides on pituitary gonadotropic function: by downregulating lhβ expression and by decreasing pituitary sensitivity to GnRH via downregulation of GnRH receptor expression. This is in good agreement with the low expression levels of gnrh-r2 in the pituitaries of males and females at the silver stage (53). The neurokinin and kisspeptin systems may thus contribute to the strong inhibitory control of eel reproductive function.

Contrary to the downregulation of both lhβ and gnrh-r2 expression, we observed no regulation of the expression of other pituitary hormones (fshβ, tshβ, and gh) by eel pituitary cells after treatment with human and eel TAC3 peptides. In carp,
homologous NKB and NKB-RP did not affect gh, lhb, fshb, tshb, somatolactin β (slβ), pomc, and gpa expression, nor GH, LH and SLβ release, by primary cultures of pituitary cells, but they did induce secretion, cell content and mRNAs of prolactin (PRL) and Slα (6). These in vitro results in the carp were in agreement with hybridization signals for neurokinin receptors, NK2 receptors on PRL cells, NK3 receptors on Slα cells, with absence of neurokinin receptor signals in other cell types, SLβ and GH cells (6). In mammals, such stimulatory role of NKB has already been reported on PRL release in rat pituitary cells (72) and on TRH-induced prl mRNA expression in somatolactotroph GH3 cell line (62). Future studies should aim at investigating other pituitary hormones (such as PRL and SL) and localization of TAC3R receptors in eel pituitary.

In this study, the four eel TAC3 peptides have a consistent effect: inhibition of lhb and gnrh-r2 expression. The biological activity and similar effect of these peptides may be related to their characteristic C-terminal tachykinin motif, and their conserved 3D α-helix structure. This may indicate a system of tachykinergic co-transmission to modulate the response, as suggested for TAC1 peptides (73). In this way, the effect of one peptide may be modulated by the co-expression of all other three, thus adjusting the response to the possible different signals.

This study addressed the effects of TAC3 peptides on pituitary gonadotropin and gnrh-r expression. Future studies may aim at investigating the possible action of TAC3 peptides on kisspeptin and kisspeptin receptors (47, 74) as well as dopamine receptors (75, 76) to further decipher TAC3 mechanisms of actions and interactions at the pituitary level. Potential interactions between tachykinin, kisspeptin, GnRH, and dopamine systems remain also to be explored at the brain level.

In conclusion, in a basal teleost, the European eel, we identified two tac3 genes encoding four TAC3 peptides, NKBa which is identical to human NKB, NKB-RPa, NKBb, and NKB-RPb. Phylogeny and synteny analyses allowed us to infer that these two genes likely result from teleost-specific whole genome duplication (3R). The two paralogous genes tac3a and tac3b have been conserved in most teleost species, but large sequence divergence is observed for tac3b and recurrent events of loss of tac3b paralog have occurred independently in some teleost lineages. In the eel, the two tac3 are mainly expressed in the brain, with high levels in the diencephalon known to contain hypophysiotropic neurons. Concerning the pituitary role of the TAC3 eel peptides, our study demonstrates for the first time in vitro effects of NKBb and its related peptide. The four peptides present in the European eel are able to downregulate lhb and gnrh-r2 transcripts in primary cultures of eel pituitary cells. Thus, in the eel, NKB peptides exert a double inhibitory control on gonadotropin function, by decreasing lhb expression directly at the pituitary level, and also by reducing pituitary sensitivity to GnRH via downregulation of GnRH receptor expression. The tachykinin system, as previously shown with the kisspeptin system, may thus contribute to the strong inhibitory control of puberty observed in the European eel.

ETHICS STATEMENT

Animals were anesthetized by cold and then killed by decapitation under the supervision of authorized person (KR; No. R-75/UPMC-F1-08) according to the protocol approved by Cuvier Ethic Committee France (No. 68-027).

AUTHOR CONTRIBUTIONS

AC and HT: cloning. AC, A-GL, and SD: phylogeny and synteny analyses. BL and JL: synthesis of eel TAC3 peptides. AC and NK: 3D prediction. KR: test of peptides on primary cultures. AC: qPCR. KR and SD: design of the experiments. KR, SD, A-GL, and AC: writing of the manuscript. All the authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fendo.2018.00304/full#supplementary-material.

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