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# Characterization of tachykinin system and role in reproduction in the European eel

Aurora Campo

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*A mi madre*

## ***Abstract***

The aim of this PhD was to investigate the role of brain neuropeptides, such as neurokinin B, encoded by *tac3* gene, in the control of reproduction of an endangered species, the European eel, *Anguilla anguilla*. The sexual maturation of the eel is blocked at a prepubertal stage before the oceanic migration. Due to its basal phylogenetic position among teleosts, the eel is also a relevant model for studying molecular and functional evolution of key neuropeptides. 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 eel as in other teleosts likely result from the teleost-specific whole genome duplication (3R). Among extant teleosts, TAC3b precursor sequences exhibit more divergence than TAC3a sequences, and losses of *tac3b* paralog would have even occurred in some teleost lineages. Comparison of vertebrate TAC3 precursor sequences also confirmed that NKB-RP peptide, encoded beside NKB by *tac3* gene in actinopterygians and basal sarcopterygians, would have been lost in ancestral amniotes. Development of specific qPCR for each *tac3a* and *tac3b* paralog, allowed us to compare the tissue distribution of their transcripts. *Tac3a* and *tac3b* mRNAs showed major expression of both transcripts in the brain especially in the diencephalon. Commercially available human NKB (same sequence as eel NKBa) has been tested *in vitro* on primary culture of eel pituitary cells. Human NKB dose-dependently inhibited the expression of luteinizing hormone beta subunit (*lhβ*), while having no significant effect on the expression of other glycoprotein hormone subunits, follicle-stimulating hormone beta subunit (*fshβ*), thyroid-stimulating hormone beta subunit (*tshβ*), common glycoprotein hormone alpha subunit (*gpα*) nor on growth hormone (*gh*). Human NKB/eel NKBa also dose-dependently inhibited the expression of eel GnRH receptor 2 (*gnrh-r2*), the other GnRH receptor types being undetectable in the cell culture. The four eel peptides were then synthesized and also tested *in vitro*. They all inhibited the expression of both *lhβ* and of *gnrh-r2*. This indicates a potential dual inhibitory action of the four eel peptides at the pituitary level on both LH and GnRH receptor. These studies revealed that the tachykinin 3 system may contribute to the strong inhibitory control of sexual maturation in the European eel. Further ongoing studies concern the identification in the eel of the paralogs of the other *tac* genes (*tac1* and *tac4* paralogs), and their phylogeny analysis.

### **Host laboratory**

UMR BOREA (Biologie des ORganismes et Ecosystèmes Aquatiques),  
7 rue Cuvier CP32,  
75231 Paris Cedex 05.

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## ***Avant-propos***

This PhD was done in the frame of International Training Network (ITN) of the Marie Skłodowska-Curie Actions entitled IMPRESS, « Improved production strategies for endangered freshwater species », funded by the EU Research and Innovation Programme Horizon 2020 for the period 2015-2018.

IMPRESS aims at providing a new generation of researchers with the multidisciplinary skills and competences needed to oversee new stocking strategies for European threatened freshwater fish species (Atlantic salmon, European eel and sturgeons). The main scientific objective is to improve the methods for rearing of fish that are intended for stocking into the wild.

### **Publications**

A. Campo, A.G. Lafont, B. Lefranc, J. Leprince, H. Tostivint, N. Kamechi, S. Dufour, K. Rousseau, 2018. Tachykinin-3 genes and peptides characterized in a basal teleost, the European eel: evolutionary perspective and pituitary role; *frontiers in endocrinology*, doi: 10.3389/fendo.2018.00304

### **Publications in preparation**

A. Campo et al., Comparative evolution of tachykinin peptides and receptors with a special focus on teleosts.

### **Workshops and courses**

**4<sup>th</sup> IMPRESS workshop: Conservation and biodiversity / Productive dissemination of science (Conservatoire National du Saumon Sauvage, Chanteuges France, June 2017).**

- Introduction to wild Salmon Conservation in the Allier River (France) with special focus in social impact of conservation and scientific dissemination of science. Duration 48h. Professor Patrick Martin.

**3<sup>rd</sup> IMPRESS workshop: Broodstock management / Entrepreneurship, commercialisation and intellectual property rights (University of South Bohemia, Vodnany Czech Republic, October 2016).**

- Overview of broodstock management in ponds ecosystems. Intellectual property rights transfer market activity. Duration 78h. Professor Vojtěch Kašpar.

**Genomes et transcriptomes, approches NGS (Muséum National d'Histoire Naturelle, Paris France, Mai 2016)**

- Metadata management, quality control, filtering and transformation of data obtained in New Generation Sequencing. Biological interpretation of the results. Duration 26h. Professors Nicolas Buisine and Laurent Sachs.

**Programmation Shell pour tous (Muséum National d'Histoire Naturelle, Mai 2016)**

- Introduction to Shell syntax and programming in Linux systems for bioinformatic research and metadata management. Introduction to AWK. Duration 28h.  
Professors Loic Ponger and Nicolas Buisine.

**2<sup>nd</sup> IMPRESS workshop: Personal development and career plan (University of Valencia, Valencia Spain, March 2016)**

- Career itineraries for international scientific research and under the European Marie Curie scope in particular. Duration 25h.  
Professors Finn-Arne Weltzien and Ian Meyer.

**5<sup>th</sup> Aquagamete COST action training school: Cryopreservation of fish germ cells (University of Valencia, Valencia Spain, March 2016)**

- Cryopreservation techniques and sperm quality evaluation for fish conservation purposes. Duration 35h.  
Professors Juan Asturiano and Luz Perez.

**1<sup>st</sup> IMPRESS workshop: Genomics and bioinformatics (ZF Screens, Leiden The Netherlands, October 2015)**

- Introduction to *in silico* research using CLC Main workbench. Duration 56h.  
Professors Ron Dirks and Christian Henkel.

**Participation in international conferences**

**Taiwan-France International conference on Development, reproduction and evolution in marine organisms. NTOU, Keelung, Taiwan (July, 2016) (oral communication)**

- Characterization of novel neuropeptides in eel. Application in fish breeding programs.  
Campo A., Lafont A.G., Maugars G., Kamech, N., Rousseau K., Dufour S.

**« Ocean, Youth and Science Forum » put on by the French-American Doctoral Exchange Program on Ocean (FADEX-O) coordinated by the French Embassy in the USA. Muséum National d'Histoire Naturelle, Paris France (October, 2017) (knowledge exchange for policy making)**

- Interdisciplinary team building for science, economics and law policy making.  
All participants.

**GIA 2018. 5th International Symposium on Genomics in Aquaculture. Albufeira Portugal (March, 2018) (oral communication)**

- Evolution of tachykinin genes and peptides after teleost whole genome duplication.  
Campo A., Lafont A.G., Rousseau K., Dufour S.

**Sustaining iconic diadromous species: Potential and pitfalls of cultivation, IMPRESS Final conference: Arendal Norway (June, 2018) (oral communication)**

- Evolution of tachykinin genes and peptides after teleost whole genome duplication.  
Campo A., Lafont A.G., Rousseau K., Dufour S.

## **Participation in other meetings and seminars**

### **IMPRESS Mid-Term meeting, NorCor, Brussels Belgium (December, 2016) (oral communication)**

- Characterization of novel neuropeptides in eel. Application in fish breeding programs.  
Campo A., Lafont A.G., Maugars G., Kamech, N., Rousseau K., Dufour S.

### **NMBU Science dissemination, NMBU Oslo, Norway (April, 2016) (oral communication)**

- Study of the gonadotropin and tachykinin systems by fluorescent in situ hybridisations in prepubertal eel pituitaries.  
Campo A., Fontaine R.

### **1<sup>st</sup> IMPRESS conference, ZF-Screens, Leiden, Netherlands (October, 2015) (oral communication)**

- Characterization of novel neuropeptides in eel. Application in fish breeding programs (Pechakucha).  
Campo A., Lafont A.G., Maugars G., Kamech, N., Rousseau K., Dufour S.

### **2<sup>nd</sup> IMPRESS conference, University of Valencia, Valencia, Spain (March, 2016) (oral communication)**

- Characterization of novel neuropeptides in eel. Application in fish breeding programs.  
Campo A., Lafont A.G., Maugars G., Kamech, N., Rousseau K., Dufour S.

### **2016 BOREA Journées Scientifiques, Caen, France (July, 2016) (oral communication)**

- Introduction to thesis: Characterization of novel neuropeptides in eel. Application in fish breeding programs.  
Campo A., Lafont A.G., Maugars G., Kamech, N., Rousseau K., Dufour S.

### **3<sup>rd</sup> IMPRESS conference, University of South Bohemia, Vodnany, Czech-Republic (October, 2016) (oral communication)**

- Characterization of novel neuropeptides in eel. Application in fish breeding programs.  
Campo A., Lafont A.G., Maugars G., Kamech, N., Rousseau K., Dufour S.

### **4<sup>th</sup> IMPRESS conference, Conservatoire National du Saumon Sauvage, Chanteuges France (July, 2017) (oral communication)**

- Novel neuropeptides in the eel.  
Campo A., Rozenfield C., Lafont A.G., Rousseau K., Dufour S.

### **2017 BOREA Journées Scientifiques, Caen, France (July, 2017) (oral communication)**

- Preliminary results: Characterization of novel neuropeptides in eel. Application in fish breeding programs.  
Campo A., Lafont A.G., Maugars G., Kamech, N., Rousseau K., Dufour S.

### **Public awareness and dissemination:**

#### **The Atlantic salmon and the threats of global warming.**

- Fete de la Science 2015, Sorbonne University, Paris France (October, 2015)  
Campo A., Fleming M.S., Lafont A.G., Maugars G., Kamech N., Rousseau K., Martin P., Dufour S.

#### **Endangered Waters: saving Europe's most iconic fish species**

- EuroScientist (Sept 21, 2016)  
(<https://www.euroscientist.com/endangered-waters-saving-europes-most-iconic-fish-species/>)  
Harrison H., Mes D., Fleming M., Böhm B., Campo A., Rasheed A., Rozenfeld C., Herranz Jusgado G., Caldeira C., Gowda S., Ciani E., Santidrián Yebra-Pimentel E., Kochalski S.

### **Leadership roles**

#### **ESR leader**

- Representing 14 ESRs part of the IMPRESS European training Network of the Marie Skłodowska-Curie Actions funded by the EU Research and Innovation Programme Horizon 2020 for the period 2015-2018.  
(1 year term, October 2015-October 2016)

### **Teaching of scholars**

Conservation of European Eel, European Sturgeon and Atlantic salmon

Lectures and poster session to 70 high school students on the threats and population declines of three diadromous fish species

Lectures and posters created by the IMPRESS ESRs

Arendal High school, Arendal Norway (22<sup>nd</sup> June, 2018)



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## ***Abbreviations***

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Abbreviations	
11KT	11-keto-testosterone
17MT	17 $\alpha$ -methyltestosterone
20 $\beta$ -HSD	20 $\beta$ -hydroxy-steroid-dehydrogenase
aa	Aminoacid
AA	Arachidonic acid
AANAT	Arylalkylamine-N-acetyl-transferase
AC	Adenylyl Cyclase
ACh	Acetylcholine
ADAM	Metalloproteinase
ADH	Adenohypophysis
AMP	Adenosine Mono Phosphate
ANO1	Anoctamin 1
ANS	Autonomous Nervous System
AR	Androgen receptor
Arc	Arcuate nucleus
ATP-aseB1	Adenosine-Tri-Phosphatase B1
AVPV	Anteroventral periventricular nucleus
bp	Base pair
BPA	Brain Pituitary Adrenal axis
BPG	Brain Pituitary Gonads axis
Cb	Cerebellum
CaM	Calmodulin
cAMP	Cyclic AMP
frGnRH	Frog GnRH
cGMP	Cyclic GMP
cGnRH	Chicken GnRH
CGRP	Calcitonin-gene Related Peptide
CNS	Central Nervous System
CRH	Corticotropin Releasing Hormone
D1-R	Dopamine receptor type 1
D2-R	Dopamine receptor type 2
DA	Dopamine
DAG	Diacylglycerol
DC	Dendritic Cells
dfGnRH	Dogfish GnRH
DIO	Diodeinase enzymes
DIO2	Iodothyronine Diodeinase 2
DRG	Dorsal root ganglia
Dyn	Dynorphin
E2	Estradiol
EA	Excitatory aminoacids
EDC	Endocrine Disrupting Compounds
EGFR	Epidermal growth factor receptor

EK	Endokinin
EKA	Endokinin A
EKB	Endokinin B
EKC	Endokinin C
EKD	Endokinin D
EL	Extracellular loop
ENS	Enteric Nervous System
EOPs	Endogen opioids
ER	Estrogen receptor
ERK	Extracellular signal Regulated Kinases
ESR	Estrogen nuclear receptor
ESR	Nuclear receptor for estrogen
FSH	Follicle Stimulating Hormone
FSH $\beta$	Follicle Stimulating Hormone $\beta$ -subunit
FSH-R	FSH receptor
G	Glycine
GABA	Acid- $\gamma$ -aminobutyric
GAL	Galanin
GAL-R1	GAL receptor 1
GAL-R2	GAL receptor 2
GAL-R3	GAL receptor 3
GAP	GnRH associated peptide
gfGnRH	Goldfish GnRH
GH	Growth hormone
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMP	Guanosine Mono Phosphate
GnIH	Gonadotropin inhibiting hormone
GnRH	Gonadotropin releasing hormone
GnRH-R	GnRH receptor
GNX	Gonadectomised
GPCR	G-Protein Coupled Receptor
gpGnRH	Guinea pig GnRH
GP	Glycoprotein hormone
GP $\alpha$	Glycoprotein hormone alpha subunit
GSI	Gonadosomatic index
GTH	Gonadotropin Hormone
H	Habenula
hCG	Human Chorionic gonadotropin
HH	Hypogonadotropic hypogonadism
HK1	Hemokinin 1
hrGnRH	Herring GnRH
HYP	Hypothalamus
IBD	Inflammatory Bowel Disease
ICC	Interstitial Cell of Cajal
IL	Intracellular loop
IL1	Interleukin 1



IL3	Interleukin 3
IL8	Interleukin 8
Inv-TAC	Invertebrate tachykinin
IP	Inositol phosphate
IP3	Inositol triphosphate
Kiss	Kisspeptin
KissR	Kisspeptin Receptor
KOR	$\kappa$ -receptor
Kp52	Kisspeptin-52
Kp54	Kisspeptin-54
L-DOPA	L-3,4-dihydroxyphenylalanine
IGnRH-I	lamprey GnRH-I
IGnRH-III	lamprey GnRH-III
LH	Luteinizing Hormone
LH $\beta$	Luteinizing Hormone $\beta$ -subunit
LH-R	LH receptor
LX	Leukotriene
MAPK	MAP Kinase
MB	Midbrain
mdGnRH	Medaka GnRH
mGnRH	Mammalian GnRH
ML	Maximum Likelihood
MLC	Myosin Regulatory Light Chain
NANC	Nonadrenergic noncholinergic neurotransmission
NF- $\kappa$ B	pro-nuclear factor kappa B
NGF	Nerve Growth Factor
NK1R	Neurokinin receptor 1
NK2R	Neurokinin receptor 2
NK3R	Neurokinin receptor 3
NKA	Neurokinin A
NKB	Neurokinin B
NLT	Nuclear Lateralis Tuberosa
NO	Nitric oxide
NOS	Nitric Oxide Synthase
NPK	Neuropeptide K
NPOav	Anteroventralis nucleus preopticus
NPY	Neuropeptide Y
NPYR	NPY receptor
NP $\gamma$	Neuropeptide gamma
NV-TAC	Cnidarian tachykinin
OB	Olfactory bulb
OP	Olfactory placode
OT	Optic Tectum
P	Phosphorylation
PBDE	Polybrominated Diphenyl Ether
PCB	Polychlorinated Biphenyls

PCE	Pituitary Carp Extract
PI	Phosphoinositide
pit	Pituitary
PKA	Protein Kinase A
PKC	Protein Kinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
PLD	Phospholipase D
PNS	Parasympathetic Nervous System
POA	Preoptic area
PPTA	Pre-pro-tachykinin A
PPTB	Pre-pro-tachykinin B
PPTC	Pre-pro-tachykinin C
PR	Progesterone receptor
PRL	Prolactin
pTACRP	Protostome Tachykinin Related Peptide
PYY	peptide YY
rGnRH	Rat GnRH
ROCK	Rho associated protein kinase
ROS	Reactive Oxygen Species
RPD	Rostral Pars Distalis
sbGnRH	Seabream GnRH
sGnRH	Salmon GnRH
SRC-Grb2	Growth factor receptor-bound protein 2 with Src Homology domain
SL	Somatolactin
SNS	Sympathetic Nervous System
SP	substance P
sTAC	Stomoxytachykinin
T	Testosterone
T3	Triiodothyronin
T4	Thyroxin
TAC	Tachykinin Peptide
TACR	Tachykinin Receptor
TACRP	Tachykinin Related Peptide
TEL	Telencephalon
TGF- $\beta$	Transforming growth factor beta
tGnRH-I	Tunicate GnRH-I
tGnRH-II	Tunicate GnRH-II
TH	Thyroid Hormone
TK	Tachykinin
TM	Transmembrane domains
TMD	Transmembrane domains
TNF	Tumour Necrosis Factor
TR	Thyroid hormone receptor
TRP channel	Transient receptor potential channel

TRPV	Transient receptor potential cation channel subfamily V member
TSGD	Teleost specific genome duplication
TSH	Thyroid Stimulating Hormone
TXA2	Thromboxane A2
UTACR	<i>Urechi unicintus</i> tachykinin receptor
V	Vainilloid
Vg	Vitellogenin
VIP	Vasoactive Intestinal Polypeptide
wfGnRH	Whitefish GnRH
β-END	β-endorphin

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# **Chapter I**

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## **Introduction**

# ***I. Introduction***

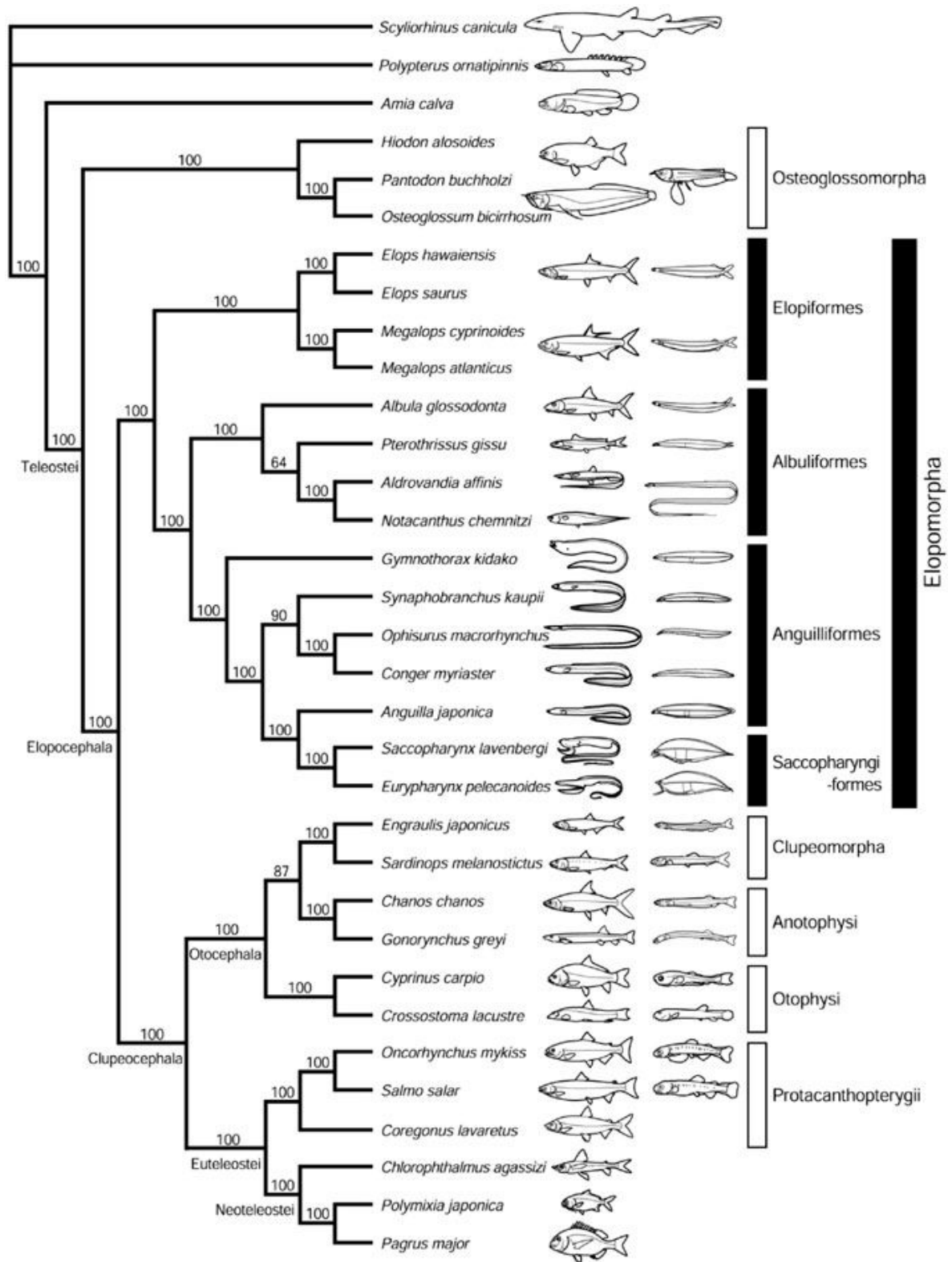
## ***1 European eel***

The European eel, *Anguilla anguilla* (Linnaeus, 1758), is a euryhaline and catadromous species with a leptocephala larva, a characteristic in common with all the species of the Elopomorpha superorder.

### ***1.1 Taxonomy***

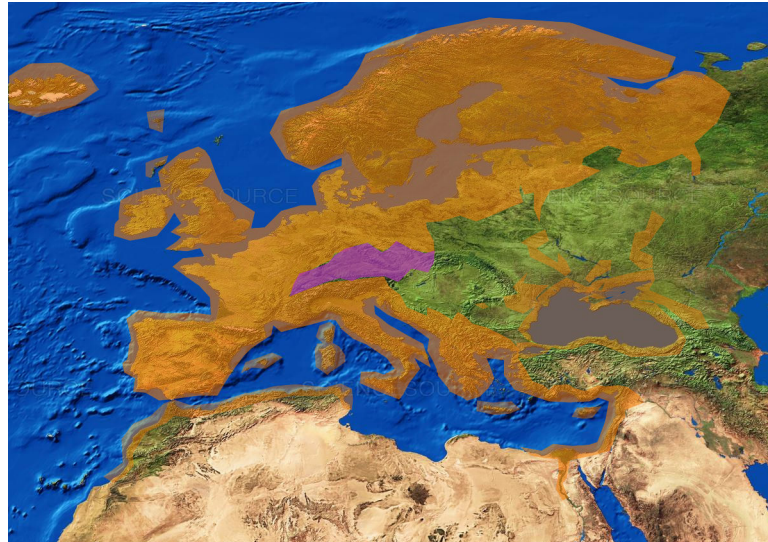
European eel taxonomic position is Class OSTEICTI; Superorder ELOPOMORPHA; Order ANGUILLIFORME; Family ANGUILLIDAE; Gender *Anguilla*; Species *A. Anguilla*.

Fifteen eel species and three subspecies have been identified in coasts and oceans all around the world except the East coast of South America (Minegishi *et al.*, 2005). The most important species in the North hemisphere are European eel, *Anguilla anguilla*, and American eel, *Anguilla rostrata*. The Japanese eel, *Anguilla japonica*, is the species from the Pacific Ocean. Eels are members of the elopomorphs, which are primitive teleost fishes that include silvery-coloured species such as tarpons (*Elopiforma*), bonefishes (*Albulidae*) or anguilliforms among many others. Their common characteristic is the development of leptocephala larvae as the first stage of their life cycle. The monophyletic origin of elopomorphs has been demonstrated by mitochondrial genomic analysis (Inoue *et al.*, 2003) and using multi-locus dataset composed of three nuclear and three mitochondrial genes (Chen *et al.*, 2014). The gender *Anguilla*, which includes the eels, belongs to the *Anguilloidei* suborder (Figure 1).



**Figure 1:** Phylogenetic position of the anguilliformes. Mitochondrial genomic evidence for the monophyly of the superorder Elopomorpha whose members all possess a *leptocephalus larvae* (Inoue *et al.*, 2003).

The genus *Anguilla* is the only genus among the elopomorphs that divides its life between the sea and freshwater environments. The European eel is distributed along the North Atlantic and Mediterranean coasts and rivers (Figure 2).



**Figure 2:** Distribution pattern of *Anguilla anguilla*. Extant eels presence area (orange) and introduced eels area (purple). Adapted manually from IUCN red list (Freyhof and Kottelat, 2010).

## **1.2 Life cycle**

The life cycle of European eel is catadromous, with an important growth phase in freshwater and a spawning in the sea. It includes two migratory phases associated with two major morphophysiological changes: larval metamorphosis and pre-puberty (Figure 3, for reviews: Otake 2003; Rousseau *et al.*, 2013).

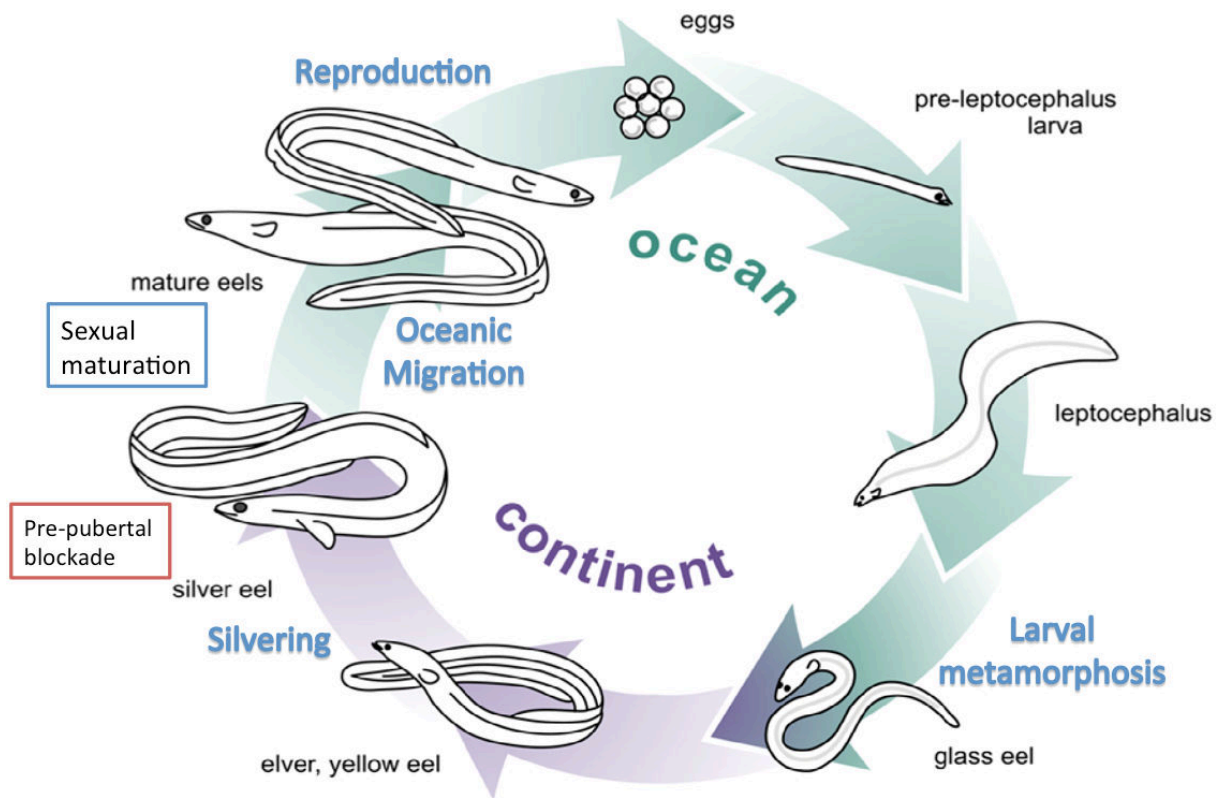


Figure 3: The life cycle of the European eel (with permission from Henckel)..

### 1.2.1 *Leptocephala* larvae and larval metamorphosis

The Danish scientist, Johannes Schmidt, started a series of expeditions in the North Atlantic Ocean for collecting leptocephala larvae, thus comparing size and abundance in each area. This data allowed deducing that the spawning areas must be located in the Sargasso sea (Schmidt, 1922). Different expeditions during the 20th century confirmed the distribution pattern of leptocephala larvae observed by Schmidt (for review: Miller *et al.*, 2015). The leptocephala larvae have oak leaf shape with a maximum size of 85mm. Leptocephala larvae below 5mm are transported passively by the currents, and after 5mm present active transport mechanisms (for review: van Ginneken and Maes 2005).

The metamorphosis from leptocephala larva to the glass eel takes place when the larvae arrive in the continental plate after a travel that takes between 8-9 months (for review: van Ginneken and Maes, 2005). Major changes are the increase of glycosaminoglycan as energy storage, changes in



internal organs structure, ossification of the vertebrae, negative phototaxis, and change of the body shape into a cylindrical one (for review: Otake 2003).

### **1.2.2 Continental growth phase**

European glass eels present estuarine and fluvial upstream recruitments that are regulated by seasonality and motivated by darkness (Tabeta and Mochioka, 2003). The larvae tend to enter in brackish waters, deltas and coastal lagoons mostly during new moon phase, even though they can be observed all year in the Mediterranean coast (Arias and Drake, 1990). This behaviour is possibly affected by temperature, salinity, turbidity, river flow, tidal cycle and has a seasonal component (Tabeta and Mochioka, 2003). Interestingly, recent studies have detected a magnetic map in glass eels that would allow them orient depending on subtle differences in magnetic field intensity and inclination angle along the migration route, that would be also linked to tidal variations (Cresci *et al.*, 2017; Naisbett-Jones *et al.*, 2017).

Along with this upstream migration behaviour, glass eel increases in body size and changes into yellow skin colour. Therefore, the upstream freshwater phase is known as the yellow eel/stage. They swim aligned beside the riverbank at the bottom, mostly with a favourable tide. Some eels remain in coastal areas such as estuaries with variable salinity, while others ascend to the beginning of the rivers.

This yellow phase lasts a particularly variable amount of time, oscillating from 6 to 12 years in males and 10 to 20 years in females (Beullens *et al.*, 1997).

### **1.2.3 Silvering as a pre-puberty event**

A second major morpho-physiological change known as silvering occurs in yellow eels after the appearance of a still unknown trigger (Dufour *et al.*, 2003; Rousseau *et al.*, 2013). Morphological external changes are the increase of the eye size and silvering of the skin. Eel skin increases thickness and reduces the yellow xantophores thus decreasing the yellow colour towards a much more silver

one (Aoyama and Miller, 2003). This phase is known as the silver eel/stage. Other important changes related to morphology and migratory behaviour are a degeneration of the digestive tract accompanied of starvation and finish of growth, activation of filament chloride cells in the gills and increase of the swim bladder to support deeper depths as found in the ocean (Aoyama and Miller 2003; Dufour *et al.*, 2003; Rousseau *et al.*, 2013; Pelster 2015). Those changes pre-adapt the eel to face the challenges in the sea environment where spawning takes place. Particularly, osmotic adaptations previous to sea migration are regulated by changes in the expression of genes related with osmoregulation in European eel (Kalujnaia *et al.*, 2007) and Japanese eel (Lai *et al.*, 2015).

Silvering occurs after activation of the gonadotropic axis indicated by an increase of the circulating sexual steroids (Han *et al.*, 2003a; Aroua *et al.*, 2005; Durif *et al.*, 2005; van Ginneken *et al.*, 2007; Dufour and Rousseau 2007; Rousseau *et al.*, 2012; Trautner *et al.*, 2017; for review: Rousseau *et al.*, 2013). Classification of sexual maturation is established from histological evaluation of oocytes. Stages I and II correspond to lipid incorporation in oocytes. Stage III corresponds to an increase in steroid production. Stage IV and V correspond to presence of nucleus or “oil droplet” in the oocytes. Since there is a marked increase in the gonadosomatic index (GSI) of 2% for females and 1% for males (Dufour *et al.*, 2003), the classification of the silvering process is therefore established according to three stages of GSI: below 0.4% for a yellow eel, between 0.4% and 1.2% for an intermediate yellow-silver eel, and above 1.2% for a silver eel. This illustrates that the progression of silvering procedure is parallel to gonad maturation as a pubertal phenomenon, but sexual maturation is blocked indicating a pre-puberty (Aroua *et al.*, 2005; Durif *et al.*, 2005; Rousseau *et al.*, 2013).

The hormones inducing the changes related to eel silvering are the sexual steroids estrogens (estradiol, E2) and androgens (testosterone, T and 11-ketotestosterone, 11KT) in all studied eel species (*Conger conger*: Sbaihi *et al.*, 2001; *A. anguilla*: Aroua *et al.*, 2005; van Ginneken *et al.*, 2007; *A. japonica*: Han *et al.*, 2003c; Jeng *et al.*, 2007; Sudo *et al.*, 2012; *A. rostrata*: Cottrill *et al.*, 2001; A.

*diffenbachii* and *A. australis*: Lokman *et al.*, 1998; and *A. australis*: Rohr *et al.*, 2001). Those increases have been observed as well on both gonadotropin mRNA in European and Japanese eel (Han *et al.*, 2003b; Aroua *et al.*, 2005; Jeng *et al.*, 2007), with an increase of expression of the gonadotropin subunits *fsh $\beta$*  at the beginning of the process and *lh $\beta$*  in the end. According to Rousseau and co-workers (2013), FSH might be related to the incorporation of lipids in the oocytes or “endogenous vitellogenesis” (stages I, II and III) while LH can be related to the second increase of sex steroid levels and late induction of Vg or “exogenous vitellogenesis” (stages IV and V). Experiments enhancing the gonadotropic axis by injection of exogenous gonadotropins (human chorionic gonadotropin, hCG or carp pituitary extract, PCE) achieved an amplification of the eye (Pankhurst 1982a ; Boëtius and Larsen 1991), increase of red muscle volume (Pankhurst 1982b), as well as a silvering and strengthening of the European eel skin (Pankhurst and Lythgoe, 1982). Intraperitoneal injection of 17 $\alpha$ -methyltestosterone (17MT), 11KT and T also enhanced the anatomical changes during silvering in eel species (Olivereau and Olivereau 1985; Boëtius and Larsen 1991; Rohr *et al.*, 2001; Aroua *et al.*, 2005).

In addition, an increase of plasma cortisol levels is detected prior to migration mostly because of starvation and apparently to mobilize metabolic energy for gonadal development and active swimming during migration (van Ginneken *et al.*, 2007). Huang and co-workers described these results as a synergistic action of cortisol and androgens in luteinizing hormone (LH) release (Huang *et al.*, 1997; Huang *et al.*, 1999; for review: Rousseau *et al.*, 2013).

#### **1.2.4 Reproductive migration**

Reproductive migration begins in the rivers and continues to the sea in autumn (Tesch and White, 2008). River migrations of European eel have been tracked by acoustic transmission between freshwater to seawater transitions. Nocturnal migrations are dominant, increasing during the three moon phases with less illumination and significant preference for migration during ebb tides (Aarestrup *et al.*, 2010; Barry *et al.*, 2016).

Even though no adults have been seen in the way to the supposed spawning area (van Ginneken and Maes, 2005), eels are likely to abandon the continental waters and swim south using probably the Canary and North-equatorial currents with a mean direction of 250° WSW at depths of about 200m-800m (van Ginneken and Maes, 2005; Tesch and White, 2008; Aarestrup *et al.*, 2009; Aoyama, 2009). A Nordic migration route for European eel was verified after observing an equal behaviour of local and translocated eels from the UK into the Baltic Sea (Westerberg *et al.*, 2014). In the case of the American eel, satellite transmission revealed sea migratory behaviour down to the Sargasso Sea (Béguer-Pon *et al.*, 2015).

The release of European silver eels in North-East of the Atlantic Ocean and nearby the Sargasso Sea confirmed daily vertical migrations in the water column nearby the spawning area (Wysujack *et al.*, 2015). These vertical migrations occurring nearby the spawning areas have been recently observed in other eel species such as *A. japonica* (Schabetsberger *et al.*, 2016; Chow *et al.*, 2015; Chen *et al.*, 2018; Higuchi *et al.*, 2018), *A. marmorata* and *A. bicolor pacifica* (Chen *et al.*, 2018). The migrations occur usually between 150-250m at night and depths to 600-800m during the day.

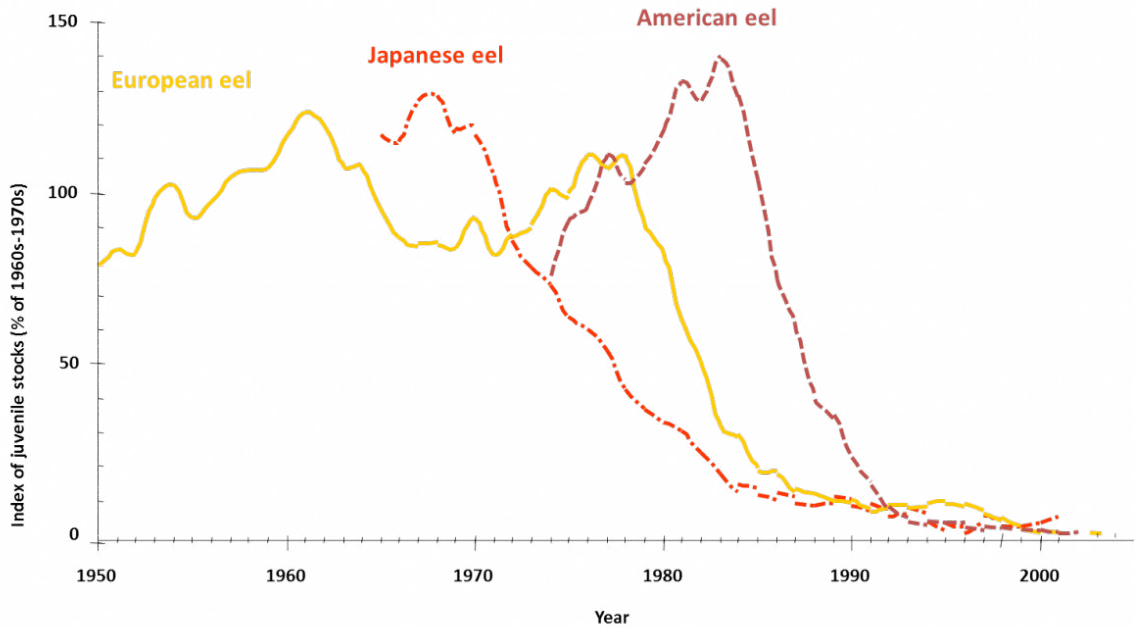
According to larval recruitment, European eels must arrive at the Sargasso Sea in March/April. They must spawn in spring or summer. Adults die supposedly after reproduction since no adults have been found in the return way (for review: Miller *et al.*, 2015).

### **1.3 Eel decline**

Eel species in the northern hemisphere experienced a decrease of the glass eel recruitment after the 80's, with a big drop to 1% of the 60's-70's population size at the beginning of the 21st century (Figure 4, Dekker *et al.*, 2003). The complexity of the life cycle and the lack of knowledge of the spawners' condition do not help in determining the cause of the population's decline. However, the growth stage and migration in the continental waters is affected by factors of anthropogenic origins, such as pollution and water quality, fisheries, habitat loss, barriers to migration and introduced

parasites (Dekker *et al.*, 2003; Dekker 2004). In addition, Oceanic alterations such Gulf Stream shifts can reduce survival leptocephala larvae during migration (Feunteun, 2002). Eel life cycle can also be altered by the presence in the environment of endocrine-disrupting compounds (EDCs), pollutants derived from the plastic use and industrial activity. For example, such components (polybrominated diphenyl ethers, PBDEs and polychlorinated biphenyls, PCBs) disrupt the expression of larval metamorphosis genes *tsh $\beta$* , *dio2* (iodothyronine deiodinase), *tra* (thyroid hormone receptor  $\alpha$ ) and *tr $\beta$*  (thyroid hormone receptor  $\beta$ ) in the European eel, that is correlated to the presence in the environment (Couderc *et al.*, 2016). In addition, exposure to these pollutants disrupted the epigenetic programming in ovarian tissue in the European eels (Pierron *et al.*, 2014). Heavy metals present in the eel environment may also affect the eel muscle and livers (south of Spain: Usero *et al.*, 2003; French Camargue: Oliveira Ribeiro *et al.*, 2005) and the cause of tumours induced by genotoxicity (Sanchez-Galán *et al.*, 2001; Oliveira Ribeiro *et al.*, 2005).

Therefore, the neuroendocrine study of the blockade of the pre-pubertal stage of silver eel is a key factor for understanding the decline and establishing patterns for European eel conservation.



**Figure 4:** Trends in glass eel recruitment. European eel (*A. anguilla*), Japanese eel (*A. japonica*) and American eel (*A. rostrata*). Average of juvenile eels recruited between 1960s and 1970s are the base for comparing the percentage of yearly recruited eels until 21<sup>st</sup> Century (Dekker *et al.*, 2003).

#### 1.4 Hormonal induction of maturation

Many laboratories have searched for environmental factors such as swimming, hydrostatic pressure, photoperiod and temperature, that can induce the sexual maturation of European eel *Anguilla anguilla* (van Ginneken *et al.* 2007; Sébert *et al.* 2007; Palstra and van den Thillart 2010). In those studies, both swimming and hydrostatic pressure increased the gonad size and induced vitellogenesis. However, none of them reached a complete maturation thus pointing towards internal neurophysiological processes.

First studies for induction of gametogenesis in European eel took place in our laboratory in 1936. The experiment supervised by Maurice Fontaine consisted of injecting pregnant human female urine extract in male eels thus achieving a complete spermatogenesis and sperm emission (Fontaine 1936). For inducing female oogenesis, carp pituitary extract was injected with positive results (Fontaine *et al.* 1964). Further experiments have confirmed that LH and FSH have a particularly weak expression in pre-pubertal silver eel (Dufour *et al.*, 1983; Aroua *et al.*, 2007). Studies on the regulation of

gonadotropin expression by our team revealed the stimulatory role of gonadotropin releasing hormone (GnRH) and the inhibitory role of dopamine. The double treatment with a GnRH agonist and an anti-dopaminergic (pimozide) could induce the synthesis and release of LH, thus producing the vitellogenesis (Dufour *et al.*, 1988; B. Vidal *et al.*, 2004).

## **1.5 Interest of European eel**

### **1.5.1 Endangered species management**

New measures for conservation of the European eel natural population and closing the life cycle in captivity are crucial steps to maintain the eel industry and reduce the socioeconomic impact of the eel critical situation.

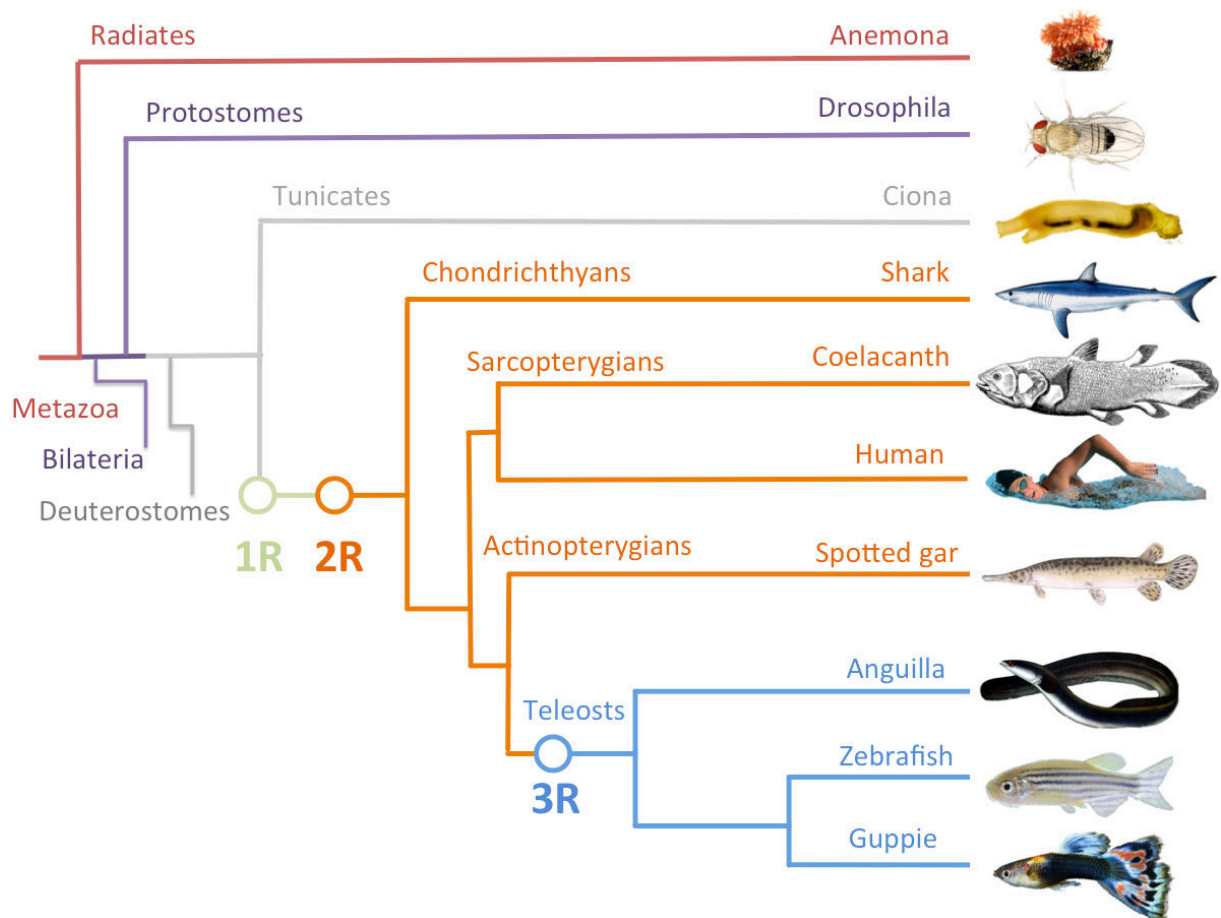
The European Commission developed eel restoration plans based on escapement to the sea of at least 40% of the biomass of adult eel together with measures for decline average market prices for eels used for restocking (Bevacqua *et al.*, 2009; Commission of the European Communities, 2012). However, no habitat restoration policies have been considered till date and monitoring of eel population does not report a population recovery (Feunteun, 2002).

Since eel life cycle has not been closed in captivity, eel exploitation is based on fattening of individuals captured in areas where stock densities were traditionally higher. The critically endangered situation of the European eel decreased the industry size and increased the market price.

### **1.5.2 Genomic interest: The base of teleost lineage**

Eels are included in the super order *Elopomorpha*, a monophyletic group placed at the base of the teleost lineage. Eels can, therefore, reflect some ancestral characteristics. Among them, it is of special interests the teleost-specific genome duplication (TSGD) ratified by primitive duplication of their hox clusters (Henkel *et al.*, 2012b).

It comes here to introduce whole genome duplications as of genetic importance in eel basal position. Genomic duplication events occurred two times during vertebrate radiation, mostly known as 1R and 2R (Dehal & Boore, 2005; Van De Peer et al., 2010). A third genomic duplication (3R) took place only in an ancestral teleost giving place to new physiological plasticity to the whole teleost lineage (Figure 5; Meyer and Van De Peer, 2005; Braasch and Postlethwait, 2012). The impact of this duplication on fish evolution relies upon which genes are retained in duplicate as paralogs and how the duplication modifies their evolutionary constraints (Brunet *et al.*, 2006).



**Figure 5:** Genome duplication events in the deuterostome radiation. 1R : First whole genome duplication event; 2R : Second whole genome duplication event ; 3R : Teleost whole genome duplication event.

Even though many teleosts have lost the half of the duplicated hox genes (Kuraku and Meyer, 2009), eel seems to keep many of them (Henkel *et al.*, 2012b) thus probably increasing the fitness adapting to many different environments during its life cycle (Pujolar *et al.*, 2015).



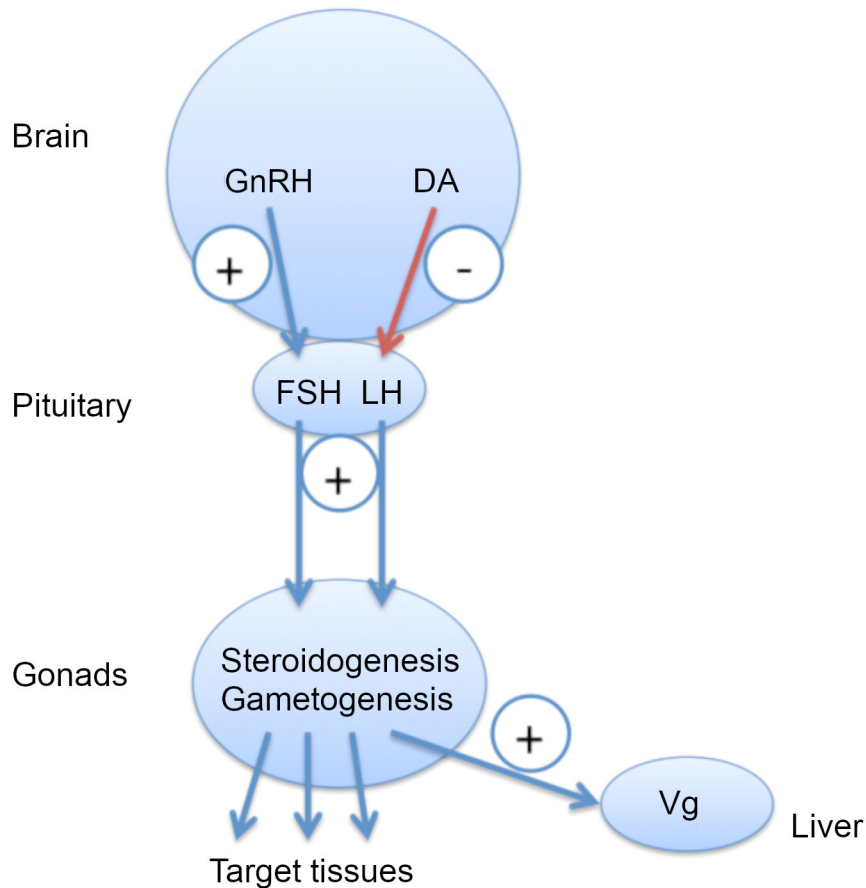
In addition to the conservation of the duplicated hox genes, eels duplicated neuropeptides and receptors are also very well preserved (Aquaporin 1: Tingaud-Sequeira *et al.*, 2008; TSH $\beta$  subunit: Manguars *et al.*, 2014; estrogen receptors: Lafont *et al.*, 2015; leptin receptors: Morini *et al.*, 2015; progesterin receptor: Morini *et al.*, 2017; GnRH-receptor I: Peñaranda *et al.*, 2013; among many others).

The duplicated and preserved genes may have a particular functionality for each environment and/or phase of the eel life cycle, including puberty and sexual maturation. Many shred of evidence of differential expression of silvering related proteins and their receptors after exposure to steroids can be found in the literature. An example is the eye rhodopsin protein for the deep-sea vision that is upregulated after injection of steroids to artificially matured Japanese eels (Zhang *et al.* 2000). Moving towards silvering reproductive neuropeptides our team found an increase in expression of one estrogen nuclear receptor (ESR1) in European eel pituitary thus pointing to a different proportion on the abundance of each receptor (Lafont *et al.*, 2015; da Silva *et al.*, 2018). In the same line, Peñaranda and co-workers found upregulated expression of GnRH receptor 1a in female eel and GnRH receptor 2 for both males and females during experimental maturation (Peñaranda *et al.*, 2013). Further general expression studies in male eel during artificial maturation have detected an upregulation of neuropeptides and G protein-coupled receptors in midbrain and forebrain suggesting a re-organization of the signalling pathways after exposure to chorionic gonadotropin. Among them, they found both up and downregulation of two different uncharacterized pro-dynorphin precursor genes that can be interpreted as dominance in the expression of a different paralog gene before and after the silvering process (Churcher *et al.*, 2014). Those pieces of evidence suggest an increase of paralog neuropeptides expressed after maturation compared to those expressed in a premature stage.

## ***2 The Brain Pituitary Gonad axis***

The hypothalamic/brain – pituitary – gonad axis (BPG) refers to the neuroendocrine axis involved in the control of reproduction in vertebrates (Figure 6). The Gonadotropin-releasing Hormone (GnRH) is the major hypothalamic peptide during puberty and sexual maturation (Yamamoto *et al.*, 1998; White & Fernald 1998; González-Martínez *et al.*, 2002; Dufour *et al.*, 2010; Zohar *et al.*, 2010). In mammals, the secreted GnRH is conducted to the pituitary by the brain-pituitary portal system, a vascular system. However, in teleost fishes GnRH neurons innervate directly the *pars distalis* of the pituitary (Yamamoto *et al.*, 1998; for review: Zohar *et al.*, 2010). GnRH interacts with the pituitary GnRH-receptors and induces the synthesis and secretion of the pituitary gonadotropins LH and FSH, which in turn stimulate the production of the sexual steroids, estrogens and androgens. In the gonads, the gonadotropins interact with specific receptors in different cell types thus producing the sex steroids testosterone (T) and estrogen (E2) (steroidogenesis) and inducing gametogenesis (for review: Zohar *et al.*, 2010). In teleosts fishes, the progestagen steroid 17K,20L-Dihydroxy-4-pregnen-3-one (17K,20L-DP) induces oocyte maturation and spermiation (Nagahama 2002).

In oviparous female animals such as birds or fishes, this BPG axis is connected to the liver (Figure 6), where chorionic proteins for the formation of the yolk, such as vitellogenin, are synthesized (Mommsen and Walsh, 1988). In teleosts, the sexual steroid E2 induce the production of vitellogenin (Vg) that takes place in the liver (Arukwe and Goksøyr, 2003).



**Figure 6:** Brain Pituitary Gonad Liver axis in teleosts (Rousseau *et al.*, 2013). GnRH: Gonadotropin releasing hormone; DA: dopamine; FSH: follicle stimulating hormone; LH: luteinizing hormone; Vg: Vitellogenin (Rousseau *et al.* 2013)

## 2.1 GnRH system

### 2.1.1 GnRH peptides

The Gonadotropin-releasing hormone (GnRH) is a decapeptide with the characteristic sequence of QxWSxGxWxPG, where x is a variable amino acid (Sherwood 1987; Lethimonier *et al.*, 2004; Millar 2005; Chen & Fernald 2008; Roch *et al.*, 2011; Roch *et al.*, 2014a).

First, GnRH was called luteinizing hormone-releasing hormone (LHRH) and was isolated from the porcine (Schally *et al.*, 1971) and ovine (Amoss *et al.*, 1971) hypothalamus. Amoss and co-workers (1971) reported that *in vitro* treatment with ovine LHRH performed on rat pituitaries induced the release of LH and FSH.

More than 20 different GnRHs have been characterized till date. The different GnRHs have been named according to the species where they were found the first time (Sherwood, 1987; Dubois *et al.*, 2002; González-Martínez *et al.*, 2002; Lethimonier *et al.*, 2004; Millar, 2005), the mammalian GnRH being the only exception to this rule.

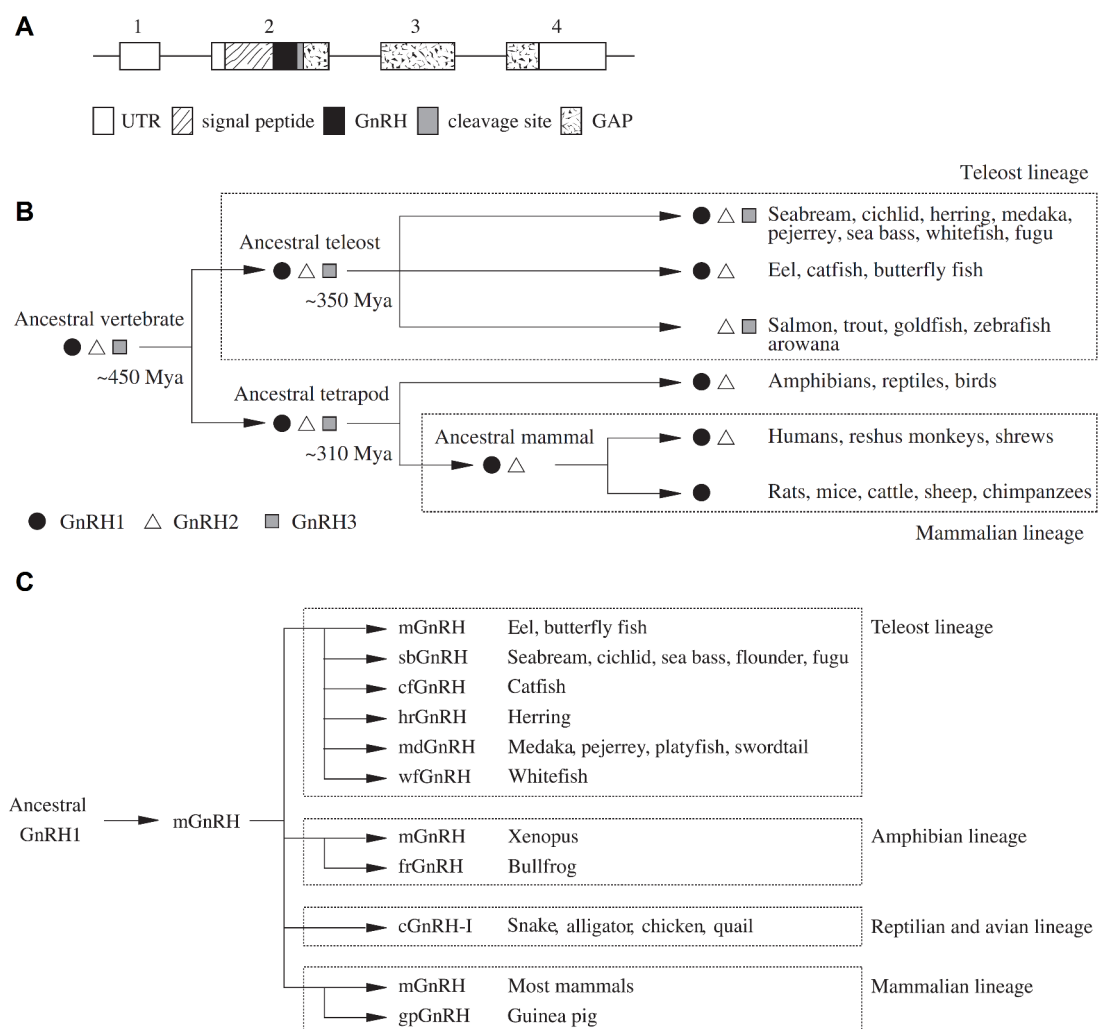
GnRH is encoded by a gene with 3 introns and 4 exons (Okubo *et al.*, 2000; Chen and Fernald, 2008; Roch *et al.*, 2011, 2014; Tostivint, 2011; X. Zhou *et al.*, 2012). GnRH-genes encode the pre-pro-GnRH that contains a signal peptide of about 23 amino acids, the GnRH decapeptide, a cleavage site (Gly-Lys-Arg) and its associated peptide of 40-60 amino acids (GAP) (Figure 7 A).

Phylogeny studies have demonstrated the existence of 4 GnRH families generated during the 1R and 2R in non-teleost vertebrates. From these, three GnRH families are preserved in teleost fishes (Lethimonier *et al.*, 2004) with duplications following the 3R teleost-specific genome duplication.

Fernald and White (1999) grouped different GnRH variants by their tissue distribution thus naming them GnRH I, II and III in agreement with phylogenetic studies (Figure 7, B and C). A fourth GnRH (GnRH-IV) have been deduced from whole genome duplication events and would be lost in the vertebrate lineage:

- GnRH-I (or GnRH): hypothalamic variant secreted by the POA GnRH-neurons, (Okubo and Nagahama, 2008). GnRH-neurons in POA innervate the teleost pituitary (Yamamoto *et al.*, 1998; for review: Zohar *et al.*, 2010). This GnRH is species-specific and in teleost corresponds mostly to seabream, medaka or salmon GnRH (Figure 7C, Soga *et al.*, 1998; Yamamoto *et al.*, 1998; Lethimonier *et al.*, 2004; Mohamed *et al.*, 2007). In the eel, it releases gonadotropins during sexual maturation thus inducing gonads development after stimulation with steroids (Montero *et al.*, 1995).

- GnRH-II: mesencephalic variant, a regulator of sexual behaviour, food intake and energy balance (Kauffman and Rissman, 2004; Temple *et al.*, 2003), also named chicken GnRH-II in teleosts (Mohamed *et al.*, 2007).
- GnRH-III: the telencephalic variant, identified only in teleosts and lost in the tetrapod lineage (Tostivint, 2011). It is not found in the eel till date (Okubo and Nagahama, 2008).
- GnRH-IV: was generated after the 2R in early vertebrates according to syntenic analysis of the paralogon region (Decatur *et al.*, 2013; Roch *et al.*, 2014), it was lost almost immediately after the second duplication and no alive vertebrate has it (Gaillard *et al.*, 2018).



**Figure 7:** Structural and phylogenetic studies on GnRH. A: structure of the GnRH pre-pro-peptide and encoded peptides: GnRH and GAP. B: Conservation of the three GnRH lineages along vertebrate radiation, being GnRH1 and GnRH2 the ones present in the eel. C: Phylogenetic tree explaining the specific names that the peptide GnRH1 receives in major vertebrate lineages (Okubo and Nagahama, 2008).

In summary, GnRH-I is expressed in POA, GnRH-II is expressed in the midbrain and GnRH-III is found in the olfactory bulbs. The conserved distribution of GnRH along vertebrates indicates that GnRH appeared very early in the evolution and has a very important function. Studies from Roch and co-workers (2014) concluded that GnRH superfamily has a monophyletic origin with a common ancestor for vertebrate and invertebrate GnRH, corazonins and adipokinetic hormones.

Each vertebrate species expresses 2 or 3 different variants of GnRH with different receptors thus illustrating the pleiotropic actions of GnRH (Lethimonier *et al.*, 2004).

### **2.1.2 GnRH receptors**

The biological activity of GnRH is mediated by a G-protein coupled receptor (GPCR) called GnRH-receptor (GnRH-R). The GPCR receptors are a family of receptors derived from the rhodopsin structure.

Teleost GnRH-R has been isolated in many species thus finding four or five GnRH-R isoforms (Gopurappilly, Ogawa and Parhar, 2013). Those isoforms have been classified into four GnRH-R major lineages or types: mammalian type I and II and non-mammalian type I and II. Non-mammalian type I is duplicated in teleosts. A non-mammalian type III has been found in teleosts and is grouped together with mammalian type II. These four types of GnRH further subdivide into six classes:

- Non-mammalian type I: teleost GnRH-Rn1 (class 1) and GnRH-Rn1b (class 2)
- Non-mammalian type II: teleost GnRH-Rn2 (class 3)
- Non-mammalian type III/mammalian type II: teleost GnRH-Rn3 (class 4) and GnRH-Rn3b/mammalian GnRH-Rm2 (class 5)
- Mammalian type I: mammalian GnRH-Rm1 (class 6)

## **2.2 Dopaminergic system**

Dopamine (DA) is an organic chemical of the catecholamine and phenylalanine families synthesized from the aminoacid L-DOPA or L-3,4-dihydroxyphenylalanine, which is synthesized from the aminoacid tyrosine. The aminoacid is processed in the dopaminergic neurons by tyrosine-hydroxylase and transformed into L-dihydroxy-phenylalanine (L-DOPA). Finally, the enzyme DOPA-decarboxylase transforms it into dopamine (Wahbe *et al.*, 1982; Gjedde *et al.*, 1991).

Dopamine is a multifunctional neurotransmitter acting mostly on the pituitary and in mammals particularly on prolactin secretion (Enjalberts *et al.*, 1986; Elsholtz *et al.*, 1991; Bole-Feysot *et al.*, 1998). However, in other sarcopterygians such as amphibians (Sotowska-Brochocka *et al.*, 1994; Trudeau *et al.*, 2010) and actinopterygians such sturgeons (Pavlick and Moberg, 1997), dopamine is characterized as a strong inhibitor of the BPG axis. In teleosts, DA inhibits basal and GnRH-stimulated LH release in a wide range of teleosts including cyprinids (Lin *et al.*, 1988; Fontaine *et al.*, 2013), silurids (De Leeuw *et al.*, 1986), salmonids (Saligaut *et al.*, 1999), cichlids (Yaron *et al.*, 2003; Bryant *et al.*, 2016) and also in the European eel (Dufour *et al.*, 1988; Vidal *et al.*, 2004; Levavi-Sivan 2004b; for reviews: Dufour *et al.*, 2005; Dufour *et al.*, 2010; Zohar *et al.*, 2010; Fontaine *et al.*, 2013).

The inhibitory activity of DA in teleosts is generally mediated by dopamine receptors type 2, or D2-R, placed in the pituitary (Chang *et al.*, 1990; Yu and Peter, 1992; Vacher *et al.*, 2000; Fontaine *et al.*, 2013). However, some teleosts use the dopamine receptor type 1, or D1-R (Roche *et al.*, 2018), thus illustrating the metabolic plasticity of teleosts BPG.

## **2.3 Pituitary gonadotropins**

The gonadotropins I (GTH-I; follicle stimulating hormone or FSH) and II (GTH-II; luteinizing hormone or LH) are glycoproteic hormones together with chorionic gonadotropin (hCG). Those peptides are characterized by post-translational glycosylations. Gonadotropic cells in the pituitary produce FSH and LH, while hCG comes from the placenta. All these peptides have two subunits  $\alpha$  and

$\beta$ ; sharing the same  $\alpha$ -subunit, the glycoprotein- $\alpha$  (GP  $\alpha$ ). The  $\beta$ -subunit peptides are specific for each hormone and are encoded by different genes: *fsh $\beta$*  and *lh $\beta$* . GP $\alpha$  is encoded in the *gp $\alpha$*  gene and this subunit is common for gonadotropins and for TSH  $\beta$ -subunit. The biological activity of gonadotropins requires the association of both subunits (Vaitutaitis *et al.*, 1970; Hagen and McNeilly, 1975), and the  $\beta$ -subunit offers the specific function to each hormone (Themmen and Huhtaniemi, 2000).

## **2.4. Sex steroids**

Gonadal/sex steroids (estrogens, androgens and progesterone) are synthesized mostly by the gonads and with less importance by adrenal glands, brain and other tissues. Steroids can act as regulators of neuronal plasticity and brain development, control of feedback loops and neuroprotection against malign agents (McEwen, 2002). Those sex steroids act through androgen, progesterone and estrogen receptors. The sex steroids regulate the gonadotropic axis with negative and positive feedback loops. In mammal juveniles, steroids induce a negative feedback loop that inhibits GnRH release (Ramirez and McCann, 1965; Goodman and Karsch, 1980; for review: Sisk and Foster, 2004). This negative feedback decreases with puberty and GnRH increases its release thus up regulating the luteinizing hormone and therefore inducing the gonads maturation (for review: Sisk and Foster, 2004). Estrogen is particularly important since it acts as a switcher thus inverting negative LH and GnRH regulation feedback loops into positive ones (Moenter *et al.*, 1990) via estrogen receptor  $\alpha$  (Scully *et al.*, 2015). However, this negative feedback may be different in fishes, being more a modulator feedback (Weltzien *et al.*, 2004).

## **2.5. Main regulators of BPG-axis**

### **2.5.1 Gonadal peptides**

The gonadal peptides, inhibins, activins and follistatins, are hormones that also regulate the LH and FSH release in the pituitary and have a paracrine action on the gonads in mammals (Ling *et al.*, 1986; Meunier *et al.*, 1988; D. Gospodarowicz and Lau, 1989; Nakamura *et al.*, 1990; Bilezikjian *et al.*,



2004; for review: Namwanje and Brown, 2016) and teleosts (zebrafish: Wu *et al.*, 2000; eel: Aroua *et al.*, 2012; for review: Petrino *et al.*, 2007). Inhibins and activins A, B and AB are dimeric proteins from the transforming growth factor  $\beta$  superfamily. Activins are dimers formed by two activin- $\beta$  subunits,  $\beta$ A and  $\beta$ B. The combination of pairs of these two subunits results in activin A ( $\beta$ A+ $\beta$ A), activin B ( $\beta$ B+ $\beta$ B) and activin AB ( $\beta$ A+ $\beta$ B). Inhibins are also dimers with one inhibin- $\beta$  subunit and one inhibin- $\alpha$  subunit. There is one inhibin- $\alpha$  subunit and there are two inhibin- $\beta$  subunits,  $\beta$ A and  $\beta$ B. The inhibin A is formed by inhibin- $\alpha$  and inhibin- $\beta$ A subunits and the inhibin B is formed by inhibin- $\alpha$  and inhibin- $\beta$ B. While the beta subunit in inhibin is more similar to the beta subunit in activin, the alpha subunit is more divergent (Burger, 1988; Robertson *et al.*, 2004). These protein complexes are produced in many tissues but especially in the ovary and pituitary. Activins stimulate directly FSH secretion but no effect on LH has been found (Ling *et al.*, 1986; Vale *et al.*, 1986). Interestingly, inhibins have the contrary effect, downregulating FSH synthesis and inhibiting the secretion (Van Zonneveld *et al.*, 2003).

Follistatins are glycoproteins with a single chain that binds to activin (Nakamura *et al.*, 1990; Robertson, 1992; Suginos *et al.*, 1993) and to inhibin (Shimonaka *et al.*, 1991). In mammals, follistatins present an alternative splicing that generates one protein of 315 aa (follistatin 315) and 288 aa (follistatin 288). They are synthesized in the pituitary and the ovary among other tissues, where they act as paracrine factors (D Gospodarowicz and Lau, 1989). Follistatins 315 and 288 have the capacity to inhibit, *in vitro* and *in vivo*, FSH release through blocking the activity of activins in rat (Nakamura *et al.*, 1990).

Activin A ( $\beta$ A+ $\beta$ A), inhibin A ( $\alpha$ + $\beta$ A) and follistatin have been found in the zebrafish ovary (Wu *et al.*, 2000). In these studies, activin and inhibin induced final oocyte maturation in a dose dependent manner. Follistatin blocked the effect of activin/inhibin and also the final oocyte maturation. Therefore, activins/inhibins have been proposed as paracrine regulators of ovarian function in fishes.

Additionally, inhibin A is upregulated by FSH, which in turn is inhibited by inhibin A thus suggesting a negative feedback loop between pituitary and teleost ovary (Poon *et al.*, 2009).

### **2.5.2 RF-amides**

This large family contains peptides with a characteristic arginine (R) followed by a phenylalanine (F) that is amidated in the C-terminal end. The first molecule of this family has been characterized by Price and Greenberg as a cardiac excitatory in the mollusc *Macrocallista nimbosa* (Price and Greenberg, 1977). Many peptides with the RF-amide motif have been characterized in other metazoans (Walker *et al.*, 2009; Osugi *et al.*, 2014, 2016; Pasquier *et al.*, 2014; Tsutsui *et al.*, 2018).

Thanks to phylogenetic studies, RFamides are classified in 5 groups (GnIH, kisspeptin, NPDF, 26RFa and PrRP (Fukusumi *et al.*, 2006; Tsutsui *et al.*, 2010)), two of which have been involved in the control of reproduction.

**GONADOTROPIN INHIBITORY HORMONE (GnIH):** is a dodecapeptide identified in birds (Tsutsui *et al.*, 2000). This peptide is also present in amphibians (fGRP and fGRP-RP-2, Tsutsui, 2009), mammals (RFRP) and invertebrates (for reviews: Kriegsfeld *et al.*, 2006, 2015, Tsutsui *et al.*, 2006, 2007, 2010, 2013, 2018; Iwasa *et al.*, 2012). In birds, this peptide is expressed mainly in hypothalamic neurons and inhibits the synthesis and release of gonadotropins by acting directly in the pituitary (Tsutsui *et al.*, 2007). In vertebrates, environmental signals such as photoperiod regulate GnIH and produce the seasonal breeding behaviour (Kriegsfeld *et al.*, 2015). In amphibians, GnIH is suggested to be more a GH-releasing factor (Ukena *et al.*, 2003).

The role in teleost is ambiguous. Studies in tilapia *Oreochromis niloticus* showed that GnIH peptides stimulate both FSH and LH and inhibits GH (Biran *et al.*, 2014b) and *lh* and *fsh* mRNA levels in goldfish *Carassius auratus* (Moussavi *et al.*, 2014). Controversially, LH is inhibited in serum of the same goldfish (Qi *et al.*, 2013), common carp *Cyprinus carpio* (Peng *et al.*, 2016) in cavefish *Astyanax altiparanae* (Branco *et al.*, 2018) and in orange spotted grouper *Epinephelus coioides* (Wang *et al.*,

2015), among many others. Interestingly, *in vitro* studies with pituitary cells from different stages of goldfish show that secretion of LH and expression of *lh β* mRNA is dissociated after administration of GnIH and depends on the status of recrudescence. In this way, while GnIH enhances the secretion of LH in pre-spawning fish, it decreases the *lh β* expression in early recrudescence, increases it at mid recrudescence to decrease it again at pre-spawning stage (Moussavi *et al.*, 2012).

In addition, a regulation of hypothalamic GnRH by GnIH during the sexual maturation has been described for goldfish (Qi *et al.*, 2013) and orange spotted grouper (Wang *et al.*, 2015). Therefore, GnIH can be a regulator of the BPG axis during sexual maturation in teleosts.

**KISSPEPTIN (Kiss):** The system of kisspeptin peptides and receptors (Kiss/KissR) was identified initially in cancer processes. A Kiss peptide, (Kiss1) has been identified as a suppressor of metastasis in a melanoma cell line and the *kiss* mRNA have been overexpressed in tumour cells with suppressed metastasis, therefore called “metastin” (Lee *et al.* 1996). The gene for the receptor *kissR* was isolated later and cloned in rat brain. It was first identified as an orphan receptor with 40% homology to Galanin (GAL) receptor that could not be activated by GAL (Lee *et al.*, 1999). Finally, in 2001, the kisspeptin peptides were associated with the Gpr54 receptor and later called KissR (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001).

Four *kiss* genes originated in the ancestral vertebrate after the two rounds of whole genome duplication events. Two *kiss* genes, *kiss1*, encoding Kiss1 peptide, and *kiss2*, encoding Kiss2 peptide, were retained in the ancestor actinopterygian after gene loss and pseudogenization events. Later, those genes duplicated with the teleost-specific whole genome duplication, but duplicates were lost immediately after, being two genes present in the eel (for review: Pasquier *et al.*, 2014). Genome duplication and gene loss events along vertebrate radiation produced a very different conservation pattern of the *kissR* genes. While the frog *X. tropicalis* conserved three receptors, in the bird clade they disappeared (Pasquier *et al.*, 2012). The system Kiss/KissR is characterized along all the vertebrates species with an expression that differs in location and temporal patterns, but it is

generally related to the reproduction function (Pasquier *et al.*, 2012, for review: Pasquier *et al.*, 2014).

The reproductive function of the Kiss peptides is the pulsatile release of GnRH in the blood stream in mammals (Clarke 2011; Ramaswamy *et al.*, 2011; Navarro 2012; Hanchate *et al.*, 2012). This pulse is regulated from a particular neuron in the arcuate nucleus that expresses Kiss, Neurokinin B (NKB or TAC3) and dynorphin (Dyn), and it is called KNDy neuron (Navarro 2012; Merkley *et al.*, 2012; Mittelman-Smith *et al.*, 2012; Goodman *et al.*, 2013; Ruka *et al.*, 2013; Skorupskaite *et al.*, 2014).

## **2.6 BPG axis in the eel**

The prepubertal silver eel is blocked in the sexual maturation process by many different inhibitory mechanisms thus reducing the number of GnRH receptors (GnRH-Rs) and pituitary gonadotropins released (Dufour *et al.*, 1988; Vidal *et al.*, 2004; Sébert *et al.*, 2008b; Peñaranda *et al.*, 2013; Jolly *et al.*, 2016; Pasquier *et al.*, 2018).

Two different GnRH forms have been characterized in European eel (Dufour *et al.*, 1993b; Montero *et al.*, 1994) and Japanese eel (Okubo *et al.*, 1999) and three splicing variants have been found (Okubo *et al.*, 2002). One of the GnRH is similar to the mammalian (mGnRH or GnRH-I) while the other is similar to the chicken one (cGnRH or GnRH-II). GnRH-III would have been lost in the eel clade independently from the other teleost lineages (Okubo and Nagahama, 2008). Studies determined the brain distribution of those peptides using radioimmunoassay (RIA) (Montero *et al.*, 1994). Eel GnRHs present a differential localization in the brain, with mGnRH (GnRH-I) most extended in the brain and in axonal endings in the pituitary gland (Dufour *et al.*, 1993b). GnRH-I (mammal-type) and GnRH-II (chicken GnRH) have been characterized for Japanese eel and both genes are expressed in different areas of the brain of the Japanese eel (Okubo *et al.* 1999; Okubo *et al.* 2000,

Figure 7). After artificial maturation of female eels, GnRH-I protein levels were upregulated in the hypothalamus and pituitary while GnRH-II was downregulated (Dufour *et al.*, 1993b).

Three GnRH-receptors have been recently characterized in European eel *Anguilla anguilla* by Peñaranda and co-workers in 2013, namely GnRH-RIa, GnRH-RIb, GnRH-RII. GnRH-RIa is found in the gonads and kidney, while it is not present in liver, fin, heart and gill. GnRH-RIb is present in all tissues except male liver, fin and kidney, being more expressed in females than in males for all the tissues. The highest difference between females and males is observed in the olfactory bulbs, di- and mesencephalon, and cerebellum. GnRH-RII shows also differential expression between males and females with no expression detected in female pectoral fin or male cerebellum. The highest transcript concentration is found in olfactory bulbs, telencephalon, di- and mesencephalon, and medulla oblongata in both sexes. This receptor is not present in liver, anterior and posterior kidney, heart, and gill (Peñaranda *et al.*, 2013). Peñaranda studies have demonstrated sexually differential upregulation of GnRH receptors during artificial maturation GnRH-RIa being upregulated in female and GnRH-RII upregulated in both sexes in the midbrain, with a peak of expression in the pituitary during early vitellogenesis. In the pituitary, the receptor II is upregulated in the final period of the sexual maturation in both males and females, in parallel to an upregulation of *lhβ* gonadotropin subunit (Peñaranda *et al.*, 2013).

Many experiments demonstrated that it is necessary a long-term treatment with estradiol (E2) and the combination of GnRH and DA antagonist to induce experimental maturation and therefore that steroid feedback acts also on the DA hypophysiotropic system (Dufour *et al.*, 1988, 2010; Vidal *et al.*, 2004; Weltzien *et al.*, 2006; Pasqualini *et al.*, 2009; Jolly *et al.*, 2016)

Dopamine neurons are distributed in the eel telencephalon, but only the ones in the anteroventralis Nucleus Preopticus (NPOav) innervate the pituitary *pars distalis* (Sébert *et al.*, 2008b; for review: Dufour *et al.*, 2010). As described previously for other teleosts, DA inhibits the gonadotropin production in the eel (Vidal *et al.*, 2004). DA receptors (D1A, D1B, D1C, D2A and D2B)

are identified in the European eel, but only the D2 is present in the pituitary, being the D2B ten times more expressed than the D2A (Kapsimali *et al.*, 2000; Vidal *et al.*, 2004; Pasqualini *et al.*, 2009; for review: Dufour *et al.*, 2005; Dufour *et al.*, 2010). In addition, *in situ* hybridization on pituitary cells revealed that D2B receptor is located in LH-producing cells and D2A is collocated with FSH, and DA highly inhibited basal and testosterone-stimulated *lh β* expression and less strongly basal and activin-stimulated *fsh β* expression by primary culture of eel pituitary cells (Jolly *et al.*, 2016).

In opposition to other teleosts, the kisspeptin system is inhibitory for gonadotropins in the eel. Two Kiss (Kiss1 and Kiss2) and three Kiss-receptors (KissR1, KissR2 and KissR3) have been characterized in European eel (Pasquier *et al.*, 2011, 2012, 2018). *In vitro* experiments with silver eel pituitary cell culture demonstrated that the expression of *gnrh-III* gene and *lhβ* are downregulated by heterologous and eel kisspeptins (Pasquier *et al.*, 2011, 2018). The three *kissR* genes are differentially expressed in the BPG axis and differentially regulated in experimentally matured eels, as compared to prepubertal controls (Pasquier *et al.*, 2012). *Kissr-1* mRNA was highly expressed in brain, pituitary and in both ovary and testis. Lower expression was detected in muscle, retina and fat tissue. *Kissr-2* mRNA was expressed in the brain and pituitary, with the highest concentration of mRNA in the telencephalic and di-/mes- encephalic areas. *Kissr-3* mRNA was highly expressed in the di-/mes-encephalic area and lowly expressed in the other areas of the brain as well as in the gonads, ovary and testis, and in the muscle. According to these studies, the differences in tissue distribution and regulation indicate a subfunctionalization of those receptors in the eel as an evolutionary constraint for the conservation of multiple KissR paralogs (Pasquier *et al.*, 2012).

Many stimulators of LH have been found (androgens: Huang *et al.*, 1997; Insulin-like growth factor: Huang *et al.*, 1998; cortisol: Huang *et al.*, 1999). Additionally, activins A and B upregulate *fshβ* subunit expression and inhibit the expression of *lhβ* with no effect in other pituitary hormones (Aroua *et al.*, 2012).

Levels of GnRH-I in the brain and gonadotropins in the pituitary increase under endogenous steroids, while GnRH-II decreases (Dufour *et al.*, 1993b). Experimentation with eel suggests that steroids may have a negative feedback regulation on GnRH-I controlled by administration of androgens, and a positive feedback effect on GnRH-II and GTH secretion dependent on administration of estradiol (Montero and Dufour, 1996). The positive feedback of steroids on the GnRH-I-gonadotropin axis in the eel can amplify the pubertal stimulation of gonadotropic function in these species (Montero and Dufour, 1996). Intracellular nuclear receptor 1 for estrogens (ESR1) are upregulated *in vitro* by estradiol in eel hepatocyte primary cultures (Lafont *et al.*, 2015). EST are then also related with eel sexual maturation, being the ESR1 consistently upregulated *in vivo* in female eel during induced sexual maturation (Lafont *et al.*, 2015).

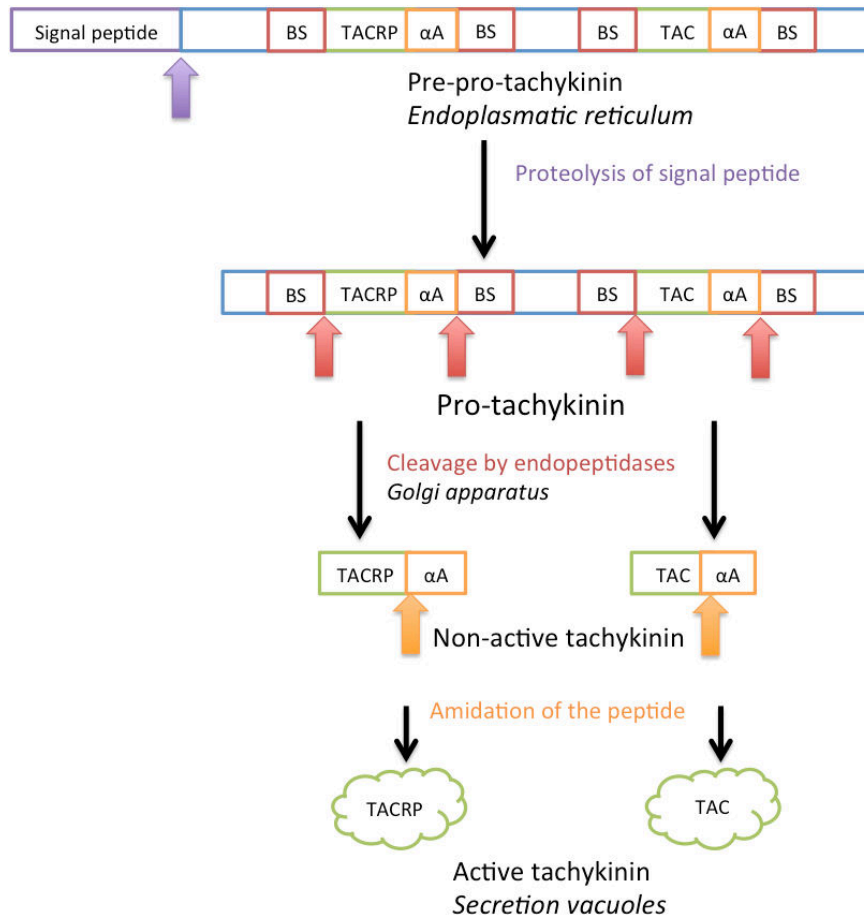
### ***3 Tachykinin peptides***

Tachykinins (TK or TAC) are members of a large family of peptides present in all the animal kingdom from cnidarian (for review: Hu *et al.*, 2014b) to a wide distribution in bilaterian (for review: Vanden Broeck *et al.*, 1999; Severini *et al.*, 2002; Pennefather *et al.*, 2004; Liu and Burcher, 2005; Van Loy *et al.*, 2010; Satake *et al.*, 2013; Hu *et al.*, 2014b). Tachykinins are brain and gut peptides, but are also found in endostyle and gonad of sea squirt (Satake *et al.*, 2013). In these tissues, they act as neurotransmitters of endocrine and local autocrine/paracrine regulations (for reviews: Severini *et al.*, 2002; Satake *et al.*, 2013). In addition, they are observed in the skin of amphibians, as well as the salivary gland of mosquito and octopus, where they serve as exocrine secretion (for review: Severini *et al.*, 2002; Liu and Burcher, 2005; Satake *et al.*, 2013; Hu *et al.*, 2014b). Their sequence is normally 10 to 14 aa long (Severini *et al.*, 2002) but exceptionally they can reach up to 45 aa by differential cleavage events as human endokinins (EK) A and B (Page *et al.*, 2003; Page, 2004) or be 7 aa long as in the oyster tachykinin related peptides (Dubos *et al.*, 2018). Their C-terminal end is a characteristic motif of 5 aa with a constant glycine in its third position: xxGxx and an  $\alpha$ -amidation (for review: Nakanishi, 1991; Almeida *et al.*, 2004; Page, 2004; Satake *et al.*, 2013; Hu *et al.*, 2014b). This C-

terminal determines the activation of the receptor (for review: Nakanishi, 1991; Maggi, 1995; Almeida *et al.*, 2004; Page, 2005).

The typical *tac* gene contains the information for the pre-pro-tachykinin peptide that includes the tachykinin peptides. The pre-pro-tachykinin uses to be large with a signal peptide at 16-30 residues at the N-terminal and can encode a variable number of tachykinin peptides. It is translated in the endoplasmatic *reticulum* and the signal peptide directs the pre-pro-peptide towards the Golgi apparatus to be secreted. The signal peptide is cleaved off by proteolysis. The spacer parts are split off by endopeptidases at specific sites of single or two basic residues in both extremes, as reported generally for neuropeptides (Harris, 1989). The inactive peptides are further amidated in the C-terminal glycine site for activation (Harris, 1989; Regoli *et al.*, 1994). After, the activated neuropeptide is packed in the secretory granules and transferred from the Golgi apparatus to the nerve terminal through the axon (Krause *et al.*, 1987; Kurtz *et al.*, 2002; Page *et al.*, 2003; for review: Pennefather *et al.*, 2004).





**Figure 8:** Processing of a tachykinin peptide. Cell processing of a vertebrate tachykinin gene; translation and peptide activation. BS: basic site for cleavage; TACRP: tachykinin-related peptide; TAC: tachykinin peptide;  $\alpha$ A:  $\alpha$ -amidation site.

The tachykinin peptides are described here for the next clades:

1. Radiata
2. Bilateria
  - a. Protostome
  - b. Deuterostome
    - i. Echinodermata
    - ii. Tunicata
    - iii. Vertebrata
      1. Agnathan
      2. Chondrichthyan
      3. Sarcopterygian
        - a. Amphibia
        - b. Sauropsida
        - c. Mammalia
      4. Actinopterygian
        - a. Chondrosteian
        - b. Holostean
        - c. Teleostean

### **3.1 Radiata**

A tachykinin-like was found by histochemistry in the *Hydra attenuata* using antibodies against the human tachykinin known as substance P (SP). The fluorescence was mostly found in the nerve cells of the ectoderm (Taban and Cathieni, 1976) and was verified as substance P-like by chromatography (Grimmelikhuijzen *et al.*, 1981). The SP-like staining was found in the ectoderm of basal disk and tentacles and not found in the foot (Taban and Cathieni, 1976; Grimmelikhuijzen *et al.*, 1981; Pierobon *et al.*, 1989). These studies detected the tachykinin peptides in nerve cells bodies (sensory neurons and ganglion cells) and in nerve fibres. Positive staining was found also in nests of differentiating small interstitial cells and in nematoblasts at various stages of differentiation (Pierobon *et al.*, 1989). Others found staining also in interstitial and gastrodermal cells (Taban and Cathieni, 1976).

The exposure to SP induced the contraction of the tentacles (Pierobon *et al.*, 1989). More recently, studies in sea anemone *Nematostella vectensis* demonstrated the presence of two tachykinin-kind peptides, namely Nv-TAC-I (YQVIFEGVR) and Nv-TAC-II (VGRTLQVGRR) (Anctil, 2009). While Nv-TAC-I presents a tachykinin-kind C-terminal motif, Nv-TAC-II is more related to collagen peptidic sequence (see collagen A1, Uniprot P02452) (for review: Hu *et al.*, 2014b). 3.2 Bilateria

#### **3.2.1 Protostome**

Two tachykinin-like peptide types, invertebrate tachykinins (Inv-TKs) and tachykinin-related peptides (TKRPs), have been identified in insects (Siviter *et al.*, 2000; Predel *et al.*, 2005), molluscs (Kanda *et al.*, 2003, 2007), echiuroid worms (Kawada *et al.*, 1999) and coelenterates (Taban and Cathieni, 1976; Anctil, 2009).

### 3.2.1.1 *Inv-TKs*

The first TK-like,eledoisin, received its name from the species it was isolated, the octopus *Eledone moschata*, in 1961 (Erspamer and Anastasi, 1961; Anastasi and Erspamer, 1962). The gene of another Inv-TK was characterized in the mosquito, *Aedes aegypti*, and named sialokinin I (Beerntsen *et al.*, 1999). Both Inv-TK were isolated from salivary glands and restricted to this tissue (Table 1). These peptides are stored in the salivary glands for exocrine secretion as toxin-like compounds (for review: Satake *et al.*, 2003, 2013; Satake and Kawada, 2006).

**Table 1:** Invertebrate tachykinins.

<b>Invertebrate tachykinins (Inv-TAC)</b>	
<i>Eledone</i>	
Ele-TAC I	EPSKDAFIGLM
Ele-TAC II	KPPSSSEFIGLM
<i>Octopus</i>	
Oct-TAC I	KPPSSSEFIGLM
Oct-TAC II	KPPSSSEFVGLM
<i>Mosquito</i>	
Sialokinin 1	NTGDKFYGLM
Sialokinin 2	DTGDKFYGLM

### 3.2.1.2 *TKRPs*

Tachykinin and tachykinin-related peptides have been found in protostomes by radioimmunoassay and immunohistochemistry: locust *Lousta migratoria* (Benedeczky *et al.* 1982), cockroach *Periplaneta americana* (Verhaert and De Loof, 1985), lobster *Panulirus interruptus* (Mancillas *et al.*, 1981), crab *Uca pucillator* (Fingerman *et al.*, 1985), among many others.

Later, sequencing techniques revealed that these mature peptides exist as tandem repeats in their precursors (Schoofs *et al.*, 1990a; Schoofs *et al.*, 1990b) and the number of repeats/copies goes from five in the fruitfly, *Drosophila melanogaster* (Siviter *et al.*, 2000) up to 13/14 in the cockroach species *Leucophaea maderae* and *Periplaneta americana* (Predel *et al.*, 2005).

Common C-terminal motive characteristics were discovered with the wide distribution of tachykinin sequences along the protostome species (Table 2): in ecdysozoa (Van Loy *et al.*, 2010b): the fruit fly *Drosophila melanogaster* (Siviter *et al.*, 2000; Winther *et al.*, 2006), and also in lophotrochozoans: worm *Urechis unitincus* (Ikeda *et al.*, 1993; Kawada *et al.*, 2002), octopus *Octopus vulgaris* (Kanda *et al.*, 2007), worm *Paranemertes sp.* (Chung *et al.*, 2015), and more recently in the oyster *Crassostrea gigas* (Dubos *et al.*, 2018a), among many others.

Protostomes TKRPs are distributed in brain and the innervations arrive till guts and oviducts (for review: Satake and Kawada, 2006). The tachykinins are also present in the cockroach *Leucophaea maderae* (Lem-TAC). LemTAC-reactive fibres are located nearby proctolin-immunoreactive fibres from the terminal ganglion to the hind gut (Nässel, 1999; Nässel and Winther, 2002). The *corpora cardiaca* in the locust is connected with the LomTAC-fibers from the lateral neurosecretory cells and related to the release of the adipokinetic hormone (Nässel *et al.*, 1995). In the fruitfly, the distribution in mid-gut endocrine cells and neuronal tissues was revealed by *in situ* hybridization (Siviter *et al.*, 2000).

*In vitro* studies demonstrated that tachykinins have a role in the protostome gut activity regulation and skeletal muscle contraction (Schoofs *et al.*, 1990a; Schoofs *et al.*, 1990b; Ikeda *et al.*, 1993; Palamiuc *et al.*, 2017). Recent studies in the oyster *Crassostrea gigas* (Dubos *et al.*, 2018) found a decrease of tachykinin receptor along the sexual maturation in the gonads, which indicates a role in sexual maturation inhibition. These functions are analogous to vertebrate tachykinins (for review: Hu *et al.*, 2014b). Interestingly, tachykinin expression increased in starved oysters, thus pointing to a possible role in feeding behaviour too (Dubos *et al.*, 2018).

**Table 2:** Protostome tachykinin related peptides.

<b>Tachykinin related peptides (pTACRP)</b>	
<i>Locust</i>	
Lom-TAC I	APSLGFHGVR
Lom-TAC II	APMRGFQSVR
Lom-TAC III	ALKGFFGTR
Lom-TAC IV	APQAGFYGVR
Lom-TAC V	GPSGFYGVV
Lom-TAC VI	APLSGFYGVR
Lom-TAC VII	APVGFYGVV
<i>Fruitfly</i>	
D-TAC I	APTSSFIGMR
D-TAC II	APLAFVGLR
D-TAC III	FIPINNRLSDVLSLEEERLRDSSLQDFFDRVAGRDGSAV
D-TAC IV	APTGFTGMR
D-TAC V	APVNSFVGMR
D-TAC VI	AALSDSYDLRGKQQRFAFDFNSKFVAVR
D-TAC VII	APNGFLGMR
<i>Echiuroid worm</i>	
Uru-TAC I	LRQSQFVGAR
Uru-TAC II	AAGMGFFGAR
Uru-TAC III	AAPSGFFGAR
Uru-TAC IV	SFSEELDEEFDMPKKPRAAYS GFFGAR
Uru-TAC V	APSMGFFGAR
Uru-TAC VI	APHMRFYGSR
Uru-TAC VII	APKMGFFGAR
<i>Oyster</i>	
Cragi-TAC I	FGFAPMR
Cragi-TAC II	ARFFGLR
Cragi-TAC III	FRFTALR

### **3.2.2 Deuterostome**

In deuterostomes, tachykinin peptides have been found in echinoderms, urochordates and vertebrates. A general motif for deuterostome-non-echinoderm tachykinins at C-Terminal is FxGLM, where x is a variable amino acid, aromatic or aliphatic (for review: Pennefather *et al.*, 2004). Some

exceptions are found, for example the human endokinins (EK) C and D that present a substitution of the final M by L (Naono *et al.*, 2007; Nishimori *et al.*, 2013).

### 3.2.2.1 Echinodermata

Tachykinins have been found in all studied echinoderms except sea urchins, indicating that asterozoa have conserved this signalling system but echinozoa has lost it (Semmens *et al.*, 2016; Zandawala *et al.*, 2017). Echinoderm tachykinins show only a single *tac* gene (Starfish *Asterias rubens*: Semmens *et al.*, 2016; ophiura *Ophiura victoriae*: Zandawala *et al.*, 2017) that have several TAC peptides encoded, being up to five in the ophiura. Echinoderm TAC peptides have modified the C-terminal end into an xxGxF motif (Table 3). The absence of central nervous system and the pentaradial symmetry of the echinoderms indicate that tachykinin function is conserved in simple nervous systems and is not related to bilaterian symmetry (Semmens *et al.*, 2016). In deuterostomes echinoderms, the tachykinin C-terminal motif is GxF where x is a leucine or an isoleucine (GLF or GIF) (Semmens *et al.*, 2016; Zandawala *et al.*, 2017). Further studies in tissue distribution of tachykinin in echinoderms will help to understand their physiological roles.

**Table 3:** Echinoderm tachykinins.

<b>Echinoderms</b>	
<i>Ophiura victoriae</i>	
Ophiura TAC I	KNNVFSAGLF
Ophiura TAC II	NGWSQGQOSGLF
Ophiura TAC III	QRWNQNQQPGLF
Ophiura TAC IV	SSGTNNMGRVRTKSSGQHVFERSGGLF
Ophiura TAC I (R)	RKNNVFSAGLF
<i>Starfish</i>	
Starfish TAC I	QLWANQQOSGLF
Starfish TAC II	GGGVPHVVFQSGGIF

### 3.2.2.2 Tunicata

In an Urochordate, the ascidian *Ciona intestinalis*, a tachykinin precursor was isolated from the neural complex and encoded two peptides, Ci-TACRP and Ci-TAC (Table 4), which share the vertebrate FXGLM motif in C-terminal (Satake *et al.*, 2004). The sequences of the two peptides are located in the same exon, indicating that both peptides are transcribed at the same moment and no alternative splicing would induce any differential transcription of both encoded peptides.

Ci-TAC precursor expression was reported to be predominant in the brain, gut and endostyle (Satake *et al.*, 2004; Kawada *et al.*, 2011), and in the ovarian (Aoyama *et al.*, 2008; Kawada *et al.*, 2017) suggesting that the tachykinins may function as brain/gut peptides and endocrine/paracrine hormones as well as be involved in oocyte maturation. These functions might have been established in the ancestral chordate (for review: Satake *et al.*, 2013).

**Table 4:** Ascidian tachykinins.

<b>Ascidian</b>	
<i>Ciona intestinalis</i>	
Ci-TACRP	QRDLYEKNKRHVRHFYGLM
Ci-TAC	SIGDQPSIFNERASFTGLM

### 3.2.2.3 Vertebrata

The evolutive storyline for tachykinin in chordates is that an ancestral *tac* gene and a *tac-receptor* gene in proto-chordates promoted four paralogs each after the two whole genome duplication rounds (1R/2R) in early vertebrates (Figure 5; Donoghue and Purnell, 2005; Dehal and Boore, 2005). This event proceeds from the possible loss of one of the four paralogs, the *tac2* gene (For review: Hu *et al.*, 2014). Further divergence caused after genome duplication events (Jaillon *et al.*, 2009) opened diverse evolutionary pathways for tachykinins. The teleost specific third genome duplication produced a tetraploidization (Figure 5; Meyer and Van De Peer, 2005; Braasch and Postlethwait, 2012) that could be followed by rediploidization and gene loss in some cases or physiology subfunctionalization in some others.

Vertebrate *tac* genes consist in five to seven exons that encode a pre-pro-tachykinin peptide. Two peptides are cleaved from the pre-pro-tachykinin (Figure 8, for review: Hu *et al.*, 2014b; Nässel 1999). The tachykinin peptide placed close to the N-terminal end will be here called TAC-related peptide or TACRP and the second peptide closer to the C-terminal extreme will be called TAC. The transcription of the pre-pro-peptide may encode one or both TAC peptides by alternative splicing events (Human, Table 7, Page *et al.*, 2009). The splicing of the gene can determine the isoform of the pre-pro-peptide, which in turn can include different lengths for the N-terminal cleavage sequence. Hence, the length of the two encoded peptides can vary in such a manner that more than two different peptides can be translated and cleaved from the same gene.

Four peptides can be translated from the gene *tac1*:

- Substance P (SP) or TAC1RP
- Neurokinin A (NKA) or TAC1
- Neuropeptide K (NPK) or TAC1-NPK
- Neuropeptide gamma (NPγ) or TAC1- NPγ

In the gene *tac3* there are two peptides encoded:

- TAC3 related peptide TAC3RP
- Neurokinin B (NKB) or TAC3.



In the gene *tac4* there are up to five possibilities for a peptide depending on the splicing. Three peptides can be translated from the TAC4RP and two from the TAC4 site:

- Hemokinin (HK) or TAC4RP-HK
- Endokinin A (EKA) or TAC4RP-EKA
- Endokinin B (EKB) or TAC4RP-EKB
- Endokinin C (EKC) or TAC4RP-EKC
- Endokinin D (EKD) or TAC4RP-EKD

The meaning of this alternative splicing is unknown, but it is likely a vertebrate characteristic since the *tac* gene in *C. intestinalis* is transcribed with both peptides included in the same exon (Satake *et al.*, 2004).

The conservation or loss of the tachykinin genes and peptides in each clade is described here from lungfishes to teleosts for illustrating the phylogenetic relations during the vertebrate radiation after genome duplication events and opening pharmacological possibilities for each peptide.

### **3.2.2.3.1 Agnathan**

A tachykinin immunoreactive to NKA antibodies was found in the brain of the sea lamprey *Petromyzon marinus* (Vaugh *et al.*, 1994) with N-terminal similar to SP (RKPHPKEWGLM). Later, the identical sequence was purified from gastrointestinal extract in the river lamprey *Lampetra fluviatilis* (David Vaugh *et al.*, 1995). A second tachykinin of 9 aa (HFDEFVGLM) more similar to NKA was found in these same extract. Further immunohistochemical analysis in the sea lamprey brain determined that tachykinins are widely distributed and there are at least two different kind of peptides (Auclair *et al.* 2004). Studies in the locomotor network of the isolated spinal chord determined that endogenous tachykinins contribute to the activity of this network (Pérez *et al.*, 2007) by reducing the calcium and potassium entry in commissural interneurons and in motor

neurons (Pérez *et al.* 2013, 2015). The number of tachykinin genes in the lamprey is still unknown and its research will contribute to construct the genetic history of the lamprey genome.

### **3.2.2.3.2 Chondrichthyan**

Tachykinin peptides have been found in elasmobranchs. A substance P immunoreactive peptide (KPRPGQFFGLM) was isolated from a brain extract from the dogfish *Scylorhinus canicula* (Waugh *et al.*, 1993). Later, Waugh and co-workers found a NKA-like peptide in skate *Raja rhina* (HKLGSFVGLM) (1994) and NPy in shark *Sphyrna lewini* (ASGPTQAGIVGRKRQKGEMFVGLM) (1995).

### **3.2.2.3.3 Sarcopterygian**

Sarcopterygians conserved the tachykinin genes and peptides. A substance P-like peptide (KPRPDEFYGLM) was found in the intestine of the lungfish *Neoceratodus forsteri* (Liu *et al.*, 2002). This peptide has a contractile action on gut circular muscle. The sequence found in lungfish is identical to a tachykinin peptide found in the intestine of cane toad *Bufo marinus* (Conlon *et al.* 1998), hence pointing to a common ancestor.

### **3.2.2.3.3.1 Amphibia**

A review has been dedicated to tachykinins in amphibians (Liu and Burcher 2005). These peptides have been identified in various anoures and one urodele. In amphibians, tachykinins have been isolated, like in other vertebrates from the intestine or the brain, but mostly from the skin (syncytial cells of the granular glands) (for review: Severini *et al.*, 2002). Many amphibian tachykinins have no known mammalian counterparts; they can be divided into aromatic and aliphatic groups (for review: Liu and Burcher 2005). The common feature of amphibian aromatic tachykinins is a Tyr residue (Y) at position 4 from the C-terminus, while amphibian aliphatic tachykinins contain either Val (V) or Ile (I) residue at position 4 from the C-terminus (for review: Liu and Burcher 2005). Most of amphibian tachykinins possess the consensus C-terminal FxGLM, where x is an aromatic (F or Y) or hydrophobic (V or I) residue, but exceptions occur as we are going to see.

### **3.2.2.3.3.1.1 Skin amphibian tachykinins**

Skin tachykinins, while conserving the C-terminal tachykinin motif, exhibit unique sequences in their N-terminus and show pharmacological profiles distinct from SP, NKA and NKB (for reviews: Severini *et al.*, 2002; Liu and Burcher 2005). They are thought to serve as exogenous factors and to have emerged from the amphibian SP, NKA and NKB genes exclusively during the evolution of amphibians (for review: Satake *et al.*, 2013). Some examples are given below. In 1977, a new peptide was isolated from the skin of the amphibian, the African frog *Kassina senegalensis*, and named kassinin (Anastasi *et al.*, 1977). This dodecapeptide (DVPKSDQFVGLM) possesses the characteristic C-terminal FVGLM but differs in the aa composition in its N-terminus (Anastasi *et al.*, 1977). Hylambatin (DPPDPDRFYGMM), isolated from the skin of the South-African frog *Hylambates maculatus*, is a tachykinin having a GMM at C-terminal end (Yasuhara *et al.*, 1981). Peptides, isolated from the skin of the Costa Rican frog *Agalychnis callidryas* (GPPDPDRFYPGM and EPDPDRFYPGM), have either PGM at their C-terminus (Mignogna *et al.*, 1997).

### **3.2.2.3.3.1.2 Brain/gut amphibian tachykinins**

A SP-related peptide, termed bufokinin (KPRPDEFYGLM), was isolated from the gut of the toad *Bufo marinus* (Conlon *et al.*, 1998) and it is identical in African clawed frog *Xenopus laevis* (Johansson *et al.*, 2002). In *Xenopus laevis*, a second peptide was isolated from intestine (TLTTGKDFVGLM) whose N-terminal region shows no structural similarity to previously known tachykinins (Johansson *et al.*, 2002). In the European green frog *Rana ridibunda* brain, a SP-related peptide, named ranakinin (KPNPERFYGLM), as well as a NKB-related peptide (DMHDFVGLM), were isolated (O'Harte *et al.*, 1991). In *Rana ridibunda*, a NKA-related peptide was also isolated from the intestine (HKLDSFIGLM) (Wang *et al.*, 1992b). In *Rana catesbeiana* brain and intestine, four novel tachykinins, named ranatachykinin A (KPSPDRFYGLM), B (YKSDSFYGLM), C (HNPASFIGLM) and D (KPNPERFYAPM) were isolated (Kozawa *et al.*, 1991). In the urodele, *Amphiuma tridactylum*, SP-related peptide showed many substitutions compared to mammalian SP (DNPSVGQFYGLM) and NKA-related peptide (HKDAFIGLM) showed two substitutions (David Waugh *et al.*, 1995).

### **3.2.2.3.3.1.3 Tissue distribution**

As we have seen, amphibian tachykinins were isolated from brain, gut and skin and their distribution in these organs have been well documented (for reviews: Severini et al., 2002; Liu and Burcher 2005). In the gastrointestinal tract, tachykinin-immunoreactive fibres have been observed in both the extrinsic (bufokinin and SP in the toad *Bufo marinus*: Osborne, 1986; Murphy and Campbell, 1993; Liu et al., 2000) and intrinsic neurons (SP/NKA in *Xenopus laevis*: Johansson et al., 2002). In amphibians, tachykinin-immunoreactivity have been reported in endocrine cells (*Hyla arborea*, *Ambystoma mexicanum*, *Cynops hongkongensis* and *Atelopus oxyrhynchus*: Buchan, 1986; salamanders *Necturus*: Holmgren, 1985; *Bufo marinus*: Liu et al., 2000; *Xenopus laevis*: Johansson et al., 2002). In the endocrine cells, amphibian tachykinin have contractile effects on smooth muscle. Amphibian tachykinins have also been detected in cardiovascular system, adrenal gland, bladder and lung (for review: Liu and Burcher 2005).

Further research in the amphibian genomes will provide information about the total duplicated tachykinin genes and peptides that occur in this clade and their possible functions.

### **3.2.2.3.3.2 Sauropsida**

#### **3.2.2.3.3.2.1 Tachykinin 1**

In sauropsids, tachykinin peptides encoded by *tac1* gene (SP, NKA) have been characterized (Table 5). In the chicken, antisera raised against N-terminal and C-terminal regions of SP and against C-terminal region of NKA were used to isolate tachykinin-like immunoreactive peptides from extracts of small intestine (Conlon et al., 1988). In 1992, Wang and collaborators isolated, using the same antisera, SP and NKA from brain of alligator *Alligator mississippiensis* (Wang et al., 1992a). In the Burmese python, *Python molurus*, tachykinin-like immunoreactive peptides isolated from intestine were also three: SP, NKA and NPY (Conlon et al., 1997), from which NKA was identical to

human/chicken/alligator NKA. Finally, SP and NPy were isolated from the intestine of a tortoise, *Gopherus agassizii* (Wang et al 1999).

**Table 5:** Tachykinin 1 peptides in sauropsids

<b>Tac1 derived peptides</b>	
<i>Chicken</i>	
TAC1RP (SP)	RPRPQQFFGLM
TAC1 (NKA)	HKTDSFVGLM
<i>Alligator</i>	
TAC1RP (SP)	RPRPQQFFGLM
TAC1 (NPy)	DAGYGQISHKRHK TDSFVGLM
TAC1 (NKA)	HKTDSFVGLM
<i>Python</i>	
TAC1RP (SP)	RPRPQQFYGLM
TAC1 (NPy)	DAGYSPLSHKRHK TDSFVGLM
TAC1 (NKA)	HKTDSFVGLM
<i>Tortoise</i>	
TAC1RP (SP)	RPRPQQFYGLM
TAC1 (NPy)	DAGYSPLSHKRHK TDSFVGLM
TAC1 (NKA)	HKTDSFVGLM

In the intestine of the alligator, SP-immunoreactivity was confined to the peripheral innervation with fibres in the *lamina propria* and muscle layers, and cell bodies in the submucosa and myenteric plexuses (Buchan *et al.*, 1983). In the turtle *Chrysemys scripta*, SP-like immunoreactivity was located in brain, spinal cord, dorsal root ganglion, and retina (Korte *et al.*, 1980).

SP immunoreactive nerve fibres innervate blood vessels in the chicken skin as well as being localized in both epidermis and dermis (Sann *et al.*, 1996). SP/NKA-like immunoreactive neurons were observed in crocodile (*C. porosus*) salt gland and saltwater acclimation resulted in a reduction of this immunoreactivity (Cramp *et al.*, 2007).

### 3.2.2.3.3.2.2 Tachykinin 3

Page and collaborators identified TAC3 pre-pro-peptide precursors (containing NKB peptide sequence) in two reptiles (alligator *Alligator mississippiensis* and anole lizard *Anolis carolinensis*), as

well as in one bird (chicken *Gallus gallus*) by bioinformatics database analysis (Page *et al.*, 2009). The sequence of the chicken is coincident with the alligator (DMHDFVGLM) and with human, while the lizard has an insertion of a phenylalanine (DMHDFVGLM).

### 3.2.2.3.3 Mammalia

The three tachykinin genes *tac1*, *tac3* and *tac4* are conserved in all the mammalian clade and the *tac3* gene is common to the studied mammals (Table 6). The peptides encoded in the *tac1* and *tac4* gene are divergent for some studied mammal species (Article 2, Supplementary figures 1 and 2; for review: Page, 2004).

**Table 6:** Mammalian tachykinins.

<b><i>Tac1</i> derived peptides</b>	
<i>Human</i>	
TAC1RP (SP)	RPKPQQFFGLM
TAC1 (NKA)	HKTDSFVGLM
TAC1-NPK	DADSSIEKQVALLKALYGHGQISHKRHKTDSFVGLM
TAC1-NP $\gamma$	DAGHGQISHKRHKTDSFVGLM
<b><i>Tac3</i> derived peptides</b>	
<i>Mammals</i>	
TAC3 (NKB)	DMHDFVGLM
<b><i>Tac4</i> derived peptides</b>	
<i>Human</i>	
TAC4RP-HK1	TGKASQFFGLM
TAC4RP (HK1 [4-11])	ASQFFGLM
TAC4RP-EKA	DGGEEQTLSTEAEWVIVALEEGAGPSIQLQLQEVKTGKASQFFGLM
TAC4RP-EKB	DGGEEQTLSTEAEWEGAGPISIQQLQLQEVKTGKASQFFGLM
TAC4-EKC	AYQLEHTFQGLL
TAC4-EKD	VGAYQLEHTFQGLL
<i>Rabbit</i>	
TAC4RP (EK-1)	GKASQFFGLM
TAC4 (EK-2)	VRGYQMGQRGLL
<i>Mouse</i>	
TAC4RP-HK1	(R) SRTRQFYGLM

### **3.2.2.3.3.1 Tachykinin 1**

In 1931, a substance, found in an extract of horse brain and intestine and first called « preparation/powder P », induced *in vitro* atropine-resistant contraction of the rabbit isolated intestine and lowered the arterial blood pressure of atropinized rabbit (Euler and Gaddum, 1931). Forty years will be needed to determine its chemical structure (Chang *et al.*, 1971).

Meanwhile, a new factor partially purified from bovine and rat hypothalamic tissue and named sialogen was shown to stimulate salivary secretion in rat (Leeman and Hammerschlag, 1967). In 1968, Lembeck and Starke reported that substance P was also able to stimulate salivary secretion in rats and suggested that sialogen and substance P may be the same peptide (Lembeck and Starke, 1968), confirmed later by Chang and Leeman (1970). Its primary structure was further determined as an amidated undecapeptide (Chang *et al.*, 1971) and its chemical synthesis obtained (Tregear *et al.*, 1971) in the same volume of Nature in 1971 (Table 6).

In 1983, two studies led to the identification of a novel peptide with a sequence homologous to that of amphibian kassinin and SP (Kimura *et al.*, 1983; Nawa *et al.*, 1983). The first one isolated this peptide from an extract of porcine spinal cord and named it neurokinin  $\alpha$  (Kimura *et al.* 1983). In 1984, a novel mammalian tachykinin designated neuromedin L was isolated from porcine spinal cord by using bioassay for a tachykinin-like effect on contractility of smooth muscle preparation from guinea pig ileum (Minamino *et al.*, 1984). Neuromedin L, substance K and neurokinin  $\alpha$  correspond to the same peptide that will be named neurokinin A (NKA, Table 6).

Two biologically active N-terminally extended forms of NKA were later identified (Table 6) and named neuropeptide K (NPK, Tatemoto *et al.*, 1985) and neuropeptide  $\gamma$  (NP $\gamma$ , Kage *et al.*, 1988).

The *tac1* gene consists of seven exons and there are different alternative splicing that produce different pre-pro-tachykinins. The  $\beta$  PPT-A is the pre-pro-tachykinin peptide encoded in the seven exons, while  $\alpha$  PPT-A lacks exon 6,  $\gamma$  PPT-A exon 4 and  $\delta$  PPT-A exons 4 and 6 (Table 7, for reviews:

Pennefather *et al.*, 2004; Page *et al.*, 2009). SP is encoded in exon 3; NKA in exon 6; NP $\gamma$  in exons 3, 5 and 6; NPK in exons 3, 4, 5 and 6 (bovine: Nawa *et al.*, 1984; human: Harmar *et al.*, 1986; Harmar *et al.*, 1990, ; rat: Carter and Krause, 1990; For reviews: Nakanishi 1986; Pennefather *et al.*, 2004).

**Table 7:** Human alternative splice events in tachykinin genes.

Gene name	Chromosome location	Isoform	Accession number NCBI	Encoded peptides
<i>tac1</i>	Ch. 7	$\alpha$	NM_013996	SP
		$\beta$	NM_003182	SP and NKA (NPK)
		$\gamma$	NM_013997	SP and NKA (NP $\gamma$ )
		$\delta$	NM_013998	SP
<i>tac3</i>	Ch. 12	$\alpha$	AF537115	NKB
		$\beta$	AF537118	NKB
		$\gamma$	AF537121	Unknown
<i>tac4</i>	Ch. 17	$\alpha$	NM_170685	EK-A and EK-C
		$\beta$	-	EK-B and EK-D
		$\gamma$	-	EK-B
		$\delta$	-	EK-B

The first study to compare the tissue distribution of  $\alpha$ -*tac1* and  $\beta$ -*tac1* isophorm mRNAs was performed in bovine by Nawa and collaborators in 1984. While  $\alpha$ -*tac1* mRNAs are mainly observed in the nervous system,  $\beta$ -*tac1* mRNAs are found in both nervous system and peripheral tissues (Nawa *et al.*, 1984). Highest expression of *tac1* mRNA was reported in brain, heart, spleen, salivary, thyroid and adrenal glands, while a minor expression was observed in cerebellum, kidney, prostate, skeletal muscle, testis, trachea, uterus and bone marrow (Pinto *et al.*, 2004). *Tac1* mRNA was undetectable in thymus, fetal and adult liver, lung and placenta (Pinto *et al.*, 2004).

Both SP and NKA were found in rat primary sensory neurons (Dalsgaard *et al.*, 1985) and splice variants  $\gamma$ -*tac1* including TAC1RP and TAC1, and  $\delta$ -*tac1* encoding only TAC1RP have also been identified in rat (Sivam *et al.*, 1987; Carter and Krause, 1990).



In the pituitary, high levels of *tac1* mRNA was reported in rat (Jonassen and Leeman, 1991). SP and NKA but no NPK in rat anterior pituitary (Brown *et al.*, 1990).

### **3.2.2.3.3.2 Tachykinin 3**

In 1983, two Japanese laboratories described the isolation from porcine spinal cord and primary structure of a novel tachykinin peptide (Kangawa *et al.*, 1983; Kimura *et al.*, 1983). This peptide possessed structural homology to tachykinin (especially to amphibian kassinin) and had the ability to induce contraction of the guinea pig ileum as SP. Kimura and collaborators named it neurokinin  $\beta$ , while Kangawa chose neuromedin K. The structure and expression of the gene for the bovine neuromedin K precursor (preprotachykinin B or PPT-B) were reported 3 years later (Kotani *et al.*, 1986). cDNA encoding *tac3* was cloned and sequenced in rat (Bonner *et al.*, 1987), mouse (Kako *et al.* 1993) and human (Page *et al.*, 2000).

The tachykinin peptide derived from the *tac3* gene, called alternatively TAC3, NKB or neuromedin K is a decapeptide, with the common tachykinin sequence FXGLM, where X here is a valine. Alternative splice events have been described for this gene (Table 7, Page *et al.*, 2009). During some time, the gene *tac3* was called *tac2* in rodents (Duarte *et al.*, 2006), but it is now referred as a synonym in the bioinformatics databases such as NCBI or ENSEMBL and *tac3* is the common form used.

The *tac3* gene consists of seven exons and NKB is encoded in exon 5 (bovine: Kotani *et al.*, 1986; For review: Pennefather *et al.*, 2004).

A wide tissue distribution of *tac3* was reported in the bovine, as soon as 1986, with a high expression in the hypothalamus and intestine (Kotani *et al.*, 1986). *In situ* hybridization study in the rat brain showed that *tac3* was widely distributed in a pattern distinct from that of SP (*tac1*) (Bonner *et al.*, 1987). The large brain distribution, including KNDy neurons (Lehman *et al.*, 2010), of *tac3* has been reviewed many times (Severini *et al.*, 2002; Rance *et al.*, 2010). Using radioimmunoassay and

immunocytochemistry, Tateishi and collaborators investigated the distribution and localisation of NKB-like immunoreactivity (NKB-LI) in rat peripheral tissues (Tateishi *et al.*, 1990). According to these studies, all tissues examined contained NKB-LI, except thyroid; they found high levels of NKB-LI in oesophagus, head of pancreas, adrenal and kidney. *Tac3* expression was reported in human placenta (Page *et al.*, 2000). *Tac3* gene expression in human was also found to be strong in brain, placenta, testis and kidney, while lower in prostate, bone marrow, salivary gland, skeletal muscle, thymus and adrenal gland, and undetectable in cerebellum, liver and spleen (Pinto *et al.*, 2004). Other reproductive organs (uterus, oviduct) than placenta, prostate and gonads have been shown to express NKB (for review: Hu *et al.*, 2014b). In intestine, while works described the localization of NKB at this level, no study has yet provided evidence for the existence of *tac3* gene (for review: Severini *et al.*, 2002; Lecci *et al.*, 2006).

#### **3.2.2.3.3.3 Tachykinin 4**

In 2000, a third tachykinin gene, *tac4*, was identified in mammals, while investigating genes differentially expressed during the transition from pro-B to pre-B stages of hematopoietic cells in mouse bone marrow (Zhang *et al.*, 2000). *Tac4* mRNA was restricted to hematopoietic cells and the 11-aa peptide encoded by *tac4* was thus termed hemokinin (HK-1, table 6) (Zhang *et al.*, 2000). The *tac4* gene was then isolated from rat brain and shown to encode a predicted HK-1, identical to mouse HK-1 (Kurtz *et al.*, 2002). In human, two groups isolated and characterized *tachykinin 4* gene, also named *tac4* from hypothalamus and thymus (Kurtz *et al.*, 2002; Page *et al.*, 2003). Kurtz *et al.* (2002) found that *tac4* gene encoded a different 11-aa peptide termed human HK-1 and a truncated form HK-1(4-11), with FFGLM (instead of FYGLM) at their C-terminal (Kurtz *et al.*, 2002). In contrast, Page *et al.* (2003) identified that the *tac4* gene transcript was spliced into four different variants that encode four different peptides named endokinins (EK) (Table 6, for reviews: Patacchini *et al.*, 2004; Page *et al.*, 2006). Similarly, in rabbit, cloning of *tac4* cDNA from pooled lung, spleen and thymus, revealed a structure comparable to that of human, with alternative splicing, giving two transcripts,  $\alpha$  and  $\beta$ -*tac4* (Page 2004). Both transcripts encoded a decapeptide identical to the C-terminal of EKA

and EKB and termed EK-1 (Table 6). A second peptide, a dodecapeptide with a divergent C-terminal and named EK-2, was characterized only in the  $\alpha$ -*tac4* isomorph with a different C-terminal (QRGLL), which were characterised as EKC and EKD (Page 2004).

The *tac4* gene consists of five exons and HK-1 is encoded in exon 2 (human and rat: Kurtz *et al.*, 2002; human: Page *et al.*, 2003; For review: Pennefather *et al.*, 2004).

In mouse, *tac4* mRNAs were detected, by RT-PCR, at the highest levels in bone marrow (hematopoietic cells), at a weaker level in thymus, but were absent in brain and spleen (Zhang *et al.*, 2000). In another study, however, moderate to strong expression were reported in the brain, spleen, stomach, skin and lactating breast, while weak *tac4* signal was detected in heart, kidney, thymus, small intestine, muscle and lung (Kurtz *et al.*, 2002). Using qPCR, Duffy *et al.*, (2003) showed that *tac4* mRNA was detected in a wide range of peripheral tissues (heart, kidney, liver, lung, stomach, spleen, adrenal, gonads and uterus) and also in numerous brain regions (Duffy *et al.*, 2003). *Tac4* mRNA was also found in the uterus, granulosa cells and blastocyst-stage embryos in the mouse (Pintado *et al.*, 2003).

In human, *tac4* was weakly expressed in kidney, liver, lung, stomach, testis, placenta, prostate and fetal liver, while a stronger expression was observed in the heart, skeletal muscle, skin and thyroid, as shown by RT-PCR (Kurtz *et al.*, 2002). Using specific primers to overlap each unique exon junction, Page and collaborators showed that while  $\alpha$  and  $\beta$  had very restricted expression patterns,  $\gamma$  and  $\delta$  were ubiquitously expressed in a range of human tissues (Page *et al.*, 2003).  $\alpha$ -*tac4* was found only in the adrenal gland, fetal liver, and very weakly in the spleen, while  $\beta$ -*tac4* was observed in the heart, liver, bone marrow, prostate, adrenal gland and testis (Page *et al.*, 2003).

Even if expressed in the brain, *tac4* mRNA is found at low levels in this tissue compared to other tachykinin genes, suggesting major peripheral roles of human HK-1 and EKs.

### **3.2.2.3.4 Actinopterygian**

#### **3.2.2.3.4.1 Chondrosteian**

Tachykinin peptides are present in sturgeons. In 1999, Wang and co-workers discovered a SP-like immunoreactive peptide with the same sequence (KPKPHQFFGLM) in the sturgeon *Scaphirhynchus albus* and the paddlefish *Polyodon spathula* and NPY in sturgeon *Scaphirhynchus albus* (SSANRQITGKRQKINSFVGLM).

#### **3.2.2.3.4.2 Holostean**

A peptide with substance P-like immunoreactivity was isolated from the stomach of the bowfin *Amia calva* (SKSHQFYGLM) by Waugh (1995). Hence, tachykinins are also present in holosteans. Further genomic research will illustrate the number of genes and peptides that were preserved before the teleost whole genome duplication.

#### **3.2.2.3.4.3 Teleostean**

##### **3.2.2.3.4.3.1 Tachykinin 1**

In teleosts, a 21aa tachykinin-related peptide, carassin, was isolated from an extract of the brain in goldfish (*Carassius auratus*); this peptide was structurally related (57% homology) to mammalian NPY (Conlon *et al.*, 1990). A few years later, the goldfish *tac1* mRNA ( $\gamma$ -*tac1*) encoding carassin (NPY) as well as SP and NKA was identified, being the first non-mammalian *tac* to be reported (Lin and Peter, 1997). Goldfish SP and NKA have 63% and 80% homology to their mammalian counterparts. In trout and cod, sequences of NKA were found to be identical to the C-terminal decapeptide of carassin, while SP sequences differed only by one residue from goldfish SP (extract from brain: Jensen and Conlon, 1992; extract from intestine: Jensen *et al.*, 1993). In 1993, the same group characterized, from trout intestine, which sequence differed from carassin by two aa (Jensen 1993). In medaka, cloning of the gene *tac1* revealed two variants: one transcript variant encoded SP, carassin and NKA, while the second transcript encoded SP and an unknown NKA-extended N-terminal sequence (Suehiro *et al.*, 2009). More recently, one *tac1* gene was identified in zebrafish *Danio rerio*

(Ogawa *et al.*, 2012; Zhou *et al.*, 2012; López-Bellido *et al.*, 2013) and grass carp *Ctenopharyngodon idella* (Hu *et al.*, 2017). The 3R paralogon of *tac1* would be lost in teleost fish lineage (Zhou *et al.*, 2012).

**Table 8:** Examples of teleost tachykinins.

<b><i>Tac1</i> derived peptides</b>	
<i>Zebrafish</i>	
TAC1RP (SP)	KPRPHQFIGLM
TAC1 (NKA)	IYLNKINSFVGLM
<b><i>Tac3</i> derived peptides</b>	
<i>Zebrafish</i>	
TAC3RPa (NKFa)	YNDIDYDSFVGLM
TAC3a (NKBa)	EMHDI FVGLM
TAC3RPb (NKFb)	YDDIDYDSFVGLM
TAC3b (NKBb)	PNMNDI FVGLL
<i>Tilapia</i>	
TAC3RP (NKF)	YNDLDYDSFVGLM
TAC3 (NKB)	EMDDI FVGLM
<i>Goldfish</i>	
TAC3RPa (NKFa)	YNDIDYDSFVGLM
TAC3a (NKBa)	EMHDI FVGLM
TAC3RPb (NKFb)	YNDIDYDSFVGLM
TAC3b (NKBb)	PNMNDI FVGLL
<i>Medaka</i>	
TAC3RP (NKF)	YTDLDYDSFVGLM
TAC3 (NKB)	DMDDI FVGLM
<b><i>Tac4</i> derived peptides</b>	
<i>Zebrafish</i>	
TAC4RPa (HK)	SKSQHFHGLM
TAC4a (EK)	NKGEI FVGLM
TAC4RPb (HK)	SKSHQFYGLM
TAC4b (EK)	HKGDMFVGLM

First studies investigating the tissue distribution of SP and NKA in teleosts used immunohistochemistry with antibodies directed against mammalian tachykinins. SP-like immunoreactivity showed extensive localization throughout the brain (goldfish *Carassius auratus*: Sharma *et al.*, 1989; rainbow trout *Oncorhynchus mykiss*: Vecino *et al.*, 1989; *Apteronotus*

*leptorhynchus*: Weld and Maler, 1992; and sea bass *Dicentrarchus labrax*: Moons *et al.*, 1992). In goldfish, carassin immunoreactivity was localized in a single nucleus, the *nucleus pretectalis posterioris*, with fibres in the preoptic area (Rao *et al.*, 1996).

In goldfish, *tac1* mRNA was reported in most brain regions (olfactory bulbs, telencephalon and preoptic region, hypothalamus, optic tectum-thalamus and cerebellum-medulla), as well in peripheral tissues such as intestine, testis and pituitary (Lin and Peter, 1997). In medaka *Oryzias latipes*, *tac1* mRNA was localized by *in situ* hybridization in different parts of the brain: telencephalon, olfactory bulbs and hypothalamus (Suehiro *et al.*, 2009). *Tac1* mRNA was observed in most regions of the zebrafish brain (olfactory bulb, telencephalon, preoptic region, hypothalamus, mesencephalon and rhombencephalon) (Ogawa *et al.*, 2012), while in grass carp, high levels were detected in cerebellum, medulla oblongata and spinal cord, lower levels in telencephalon, optic tectum and hypothalamus, but no signal in the olfactory bulb (Hu *et al.*, 2017). Transcript signals for *tac1* in peripheral tissues were found in the gills, heart, intestine, liver, muscle and gonad, but not in the kidney and blood of grass carp (Hu *et al.*, 2017).

In the pituitary, carassin-immunoreactive granules were observed in some cells of proximal pars distalis and adjacent sections reacted with growth hormone and gonadotropin antibodies (Rao *et al.*, 1996). High levels of *tac1* mRNA were reported in grass carp (Hu *et al.*, 2017) and goldfish (Lin and Peter 1997) pituitary.

#### **3.2.2.3.4.3.2 Tachykinin 3**

In teleost fishes, the *tac3* gene has been characterized in zebrafish *Danio rerio* (Biran *et al.*, 2012; Zhou *et al.*, 2012; Ogawa and Parhar, 2013), Nile tilapia *Oreochromis niloticus* (Biran *et al.*, 2014a; Jin *et al.*, 2016), grass carp *Ctenopharyngodon idella* (Hu *et al.*, 2014a), goldfish *Carassius auratus* (Qi *et al.*, 2015a), striped bass *Morone saxatilis* (Zmora, T.-T. Wong, *et al.*, 2017), and orange-spotted grouper *Epinephelus coioides* (Chen *et al.*, 2018). The teleost specific whole genome duplication has led to the duplication of the gene *tac3*, leading to two genes, *tac3a* and *tac3b*. However, one

duplicated gene seems to be lost in the case of tilapia (Biran *et al.*, 2014a; Jin *et al.*, 2016), grass carp (Hu *et al.*, 2014a), striped bass (Zmora, T.-T. Wong, *et al.*, 2017) and grouper (Chen *et al.*, 2018). A related peptide to TAC3 has been found in the *tac3* gene and named neurokinin F (NKF) (zebrafish: Biran *et al.*, 2012; tilapia: Biran *et al.*, 2014; striped bass: Zmora *et al.*, 2017) or tachykinin 3 related peptide (NKBRP or TAC3RP) (carp: Hu *et al.*, 2014a; grouper: Chen *et al.*, 2018). Thus the two *tac3* genes, *tac3a* and *tac3b* encode four peptides (Table 8), namely TAC3RPa, TAC3a, TAC3RPb and TAC3b (or NKBa-13, NKBa-10, NKBb-13 and NKBb-11 according to their aa length in zebrafish: Zhou *et al.*, 2012).

Low similarity among fish TAC3 pre-pro-peptide precursors was observed, but TAC3 peptides were well conserved across fish species (zebrafish: Biran *et al.*, 2012; Zhou *et al.*, 2012; tilapia: Biran *et al.*, 2014; carp: Hu *et al.*, 2014a; goldfish: Qi *et al.*, 2015; striped bass: Zmora *et al.*, 2017; grouper: Chen *et al.*, 2018). All the encoded peptides possessed the tachykinin signature motif (FXGLM) flanked by potential dibasic cleavage sites and an adjacent glycine at the C-terminus for amidation. In this signature motif, X represents valine (V) in all fish except cichlid NKB (tilapia and other cichlids: Biran *et al.*, 2014) and cyprinid NKBRP/NKB-13 (goldfish: Qi *et al.*, 2015) for which X represents isoleucine (I). Exception was NKB encoded by *tac3b* gene in zebrafish (Zhou *et al.*, 2012; Biran *et al.*, 2012) and goldfish (Qi *et al.*, 2015), which presented a modification of that motif with FVGLL at the C-terminus.

A wide brain expression of *tac3* was also observed in teleosts (zebrafish: Biran *et al.*, 2012; Ogawa *et al.*, 2012, Zhou *et al.*, 2012; goldfish: Qi *et al.*, 2015; grass carp: Hu *et al.*, 2014a; striped bass: Zmora *et al.*, 2017; orange-spotted grouper: Chen *et al.*, 2018). In the pituitary, a weak expression of *tac3a* but no detectable expression of *tac3b* was observed in the goldfish (Qi *et al.*, 2015), while both genes were expressed in zebrafish pituitary (Biran *et al.*, 2012; Ogawa *et al.*, 2012). In teleost species, where only *tac3a* has been identified, a weak expression was found in the pituitary (orange-spotted grouper: Chen *et al.*, 2018; grass carp: Hu *et al.*, 2014a). In the ovary, weak

expression of *tac3a* (and none for *tac3b*) was found in goldfish (Qi *et al.*, 2015) and orange-spotted grouper (Chen *et al.*, 2018). 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 (Biran *et al.*, 2012; Zhou *et al.*, 2012), it was *tac3a* gene in another study (Ogawa *et al.*, 2012). Intestine expressed *tac3a* (and not *tac3b*) in zebrafish (Jakob Biran *et al.*, 2012) and goldfish (Qi *et al.*, 2014), and the single *tac3* gene (*tac3a*) in grass carp (Hu *et al.*, 2014a) and orange-spotted grouper (Chen *et al.*, 2018).

#### **3.2.2.3.4.3.3 Tachykinin 4**

By genomic data mining, Zhou and collaborators (2012) were the first, and up to now, the only group to reveal the existence of *tac4* genes in non-mammals: *tac4a* in stickleback, *tac4b* in rainbow smelt and northern pike, as well as *tac4a* and *tac4b* in zebrafish and medaka (Zhou *et al.*, 2012)

### **3.3 Physiological role in reproduction**

In vertebrates, tachykinin peptides bind to receptors belonging to the first class rhodopsin-like G-protein coupled receptors (GPCR). There are three tachykinin receptors in human, namely NK1/TACR1, NK2/TACR2 and NK3/TACR3 (for review: Maggi, 1995; Pennefather *et al.*, 2004; Steinhoff *et al.*, 2014).

Considering the large tissue distribution of tachykinins (and their receptors), one may expect involvement of these peptides in many various physiological functions. Severini and collaborators, in 2002, reviewed not less than six different systems in which regulation tachykinins were involved: cardiovascular system, gastrointestinal tract, airways system, urogenital tract, immune system and central nervous system (Severini *et al.*, 2002). They also reported a role of these peptides in pain, neurogenic inflammation and lachrymal secretion (Severini *et al.*, 2002). In this Introduction, we will focus on the involvement of tachykinins in the control of reproductive function in vertebrates.



### **3.3.1 At the central level**

#### **3.3.1.1 On GnRH**

##### **3.3.1.1.1 Tac1 peptides**

Using a double-chamber perfusion system, Ohtsuka and collaborators (1987) were able to show that SP stimulated the *in vitro* release of LH-releasing hormone by rat mediobasal hypothalamus.

A lot of *in vivo* studies investigated the effect of *tac1*-encoded peptides (SP and NKA) on LH secretion in mammals via central pathway, using intravenous or intracerebroventricular (icv) injections (for review: Debeljuk and Lasaga, 1999; Fergani and Navarro, 2017). While most of these studies reported a stimulatory effect of SP on LH release, absence of effect was also observed. Concerning NKA, stimulation, absence of effect or even inhibition was noted. This discrepancy was related to the presence or absence of sex steroids (intact or castrated animals) or to species-difference. NPK was also shown to have an effect on LH as icv injection of the peptide produced a suppression of LH release in ovariectomized rats; in contrast, injection of NKA was ineffective and of NP $\gamma$  less effective compared to NPK (Sahu and Kalra, 1992).

In 2003, Pintado and collaborators treated neonatally female mice or rats with capsaicin (that induces depletion of SP) and observed a reduced fertility. In addition, they injected tachykinin TACR3 receptor antagonist SR 142801 to female rats and showed a reduction in the litter size (Pintado *et al.*, 2003). More recently, it was reported that female *tac1*<sup>-/-</sup> mice displayed delayed puberty (Simavli *et al.*, 2015).

##### **3.3.1.1.2 Tac3 peptide (NKB)**

Mutations in the *tac3* gene or in the *tacr3* lead to hypogonadotropic hypogonadism (Guran *et al.*, 2009; Topaloglu *et al.*, 2009; Fukami *et al.*, 2010; Gianetti *et al.*, 2010; Young *et al.*, 2010; Francou *et al.*, 2011), that could reverse in adulthood (Gianetti *et al.*, 2010). Likewise, *tac3* or *tacr3* null mice showed abnormal estrous cyclicity even though still fertile (Yang *et al.*, 2012; True *et al.*, 2015).

The first analysis of TAC3 expression showed its presence in GnRH neurons indicating a possible direct effect of TAC3 on GnRH (Krajewski *et al.*, 2005). Later, further analysis in sheep have demonstrated the existence of many neurons and immunoreactive fibres to TAC3 in POA and in other hypothalamic regions as the arcuate nucleus (ARC) (Amstalden *et al.*, 2010). Moreover, in this same area, TAC3 is co-expressed with kisspeptin (Kiss) and dynorphin (Dyn) in the so-called KNDy neurons (for reviews: Lehman *et al.*, 2010). KNDy neurons project to GnRH neurons and regulate their activity (Navarro, 2012; Grachev *et al.*, 2014; Fergani and Navarro, 2017) and ablation of those neurons in female rats induces hypogonadotropic hypogonadism (Mittelman-Smith *et al.*, 2016).

*In vivo* studies in different mammals have shown the stimulatory effect of TAC3 on GnRH/LH secretion (rat: Navarro *et al.*, 2010; mice: Navarro *et al.*, 2011; 2015; sheep: Billings *et al.*, 2010; monkey: Ramaswamy *et al.*, 2010; human: (Skorupskaite *et al.*, 2017a; 2017b).

In contrast to mammals, tachykinins and kisspeptins are expressed in different neurons in teleosts (zebrafish: Ogawa *et al.*, 2012; striped bass, *Morone saxatilis*: Zmora *et al.*, 2017). However, TAC3 peptides down-regulated *kiss2* gene expression (striped bass: Zmora *et al.*, 2017), suggesting that tachykinin peptides may act indirectly on GnRH *via* the kisspeptin system, as in mammals. The expression pattern of TAC3 and TAC3RP and the paralog receptors differs consistently from the one of the mammals. TAC3 and TAC3RP are expressed in the *nuclear lateralis tuberis* (NLT), which is the teleost homologous structure to the ARC (Biran *et al.*, 2012). However, *Kiss2* expression has not been found in this area in zebrafish (Servili *et al.*, 2011). When administered *in vivo*, NKB peptides could increase gonadotropin expression and/or release (zebrafish: Biran *et al.*, 2012; tilapia: Biran *et al.*, 2014; goldfish: Qi *et al.*, 2015; NKB in grouper: Chen *et al.*, 2018). In contrast, some studies reported no effect on gonadotropin expression (tilapia: Jin *et al.*, 2016; NKB-RP in grouper: Chen *et al.*, 2018).

### **3.3.1.2 On DA**

Future studies should aim at investigating the potential regulation of dopaminergic neurons by products of tachykinin genes, especially in teleosts, amphibians and seasonal mammals for which puberty and/or adult sexual maturation is controlled by dopamine, in addition to GnRH. Indeed, many studies in mammals reported interactions between tachykinin peptides and DA at the hypothalamic level. For example, it was suggested that tachykinins could exert an inhibitory influence on PRL release by stimulating DA release at the hypothalamic level (for review: Debeljuk and Lasaga 2006); thus it could also be the case for gonadotropin release and expression.

### **3.3.2. At the pituitary level**

A direct action of tachykinins at the pituitary level is possible as *nkr/tacr* expression has been detected in pituitary cells/gonadotropes in mammals (rat: Larsen *et al.*, 1992; sheep: Dupre *et al.*, 2010) and teleosts (zebrafish: Biran *et al.*, 2012; tilapia: Biran *et al.*, 2014a; carp: Hu *et al.*, 2017). Moreover, SP/NKA/NKB fibres have been reported surrounding hypophyseal blood capillary vessels in the median eminence in mammals (monkeys: Kalil *et al.*, 2016).

#### **3.3.2.1 Tac1 peptides**

The first reported study on the *in vitro* effect of tachykinin on gonadotropins dates back to 1974. Fisher *et al.* (1974) observed that SP induced release of LH and FSH from whole rat pituitaries cultured *in vitro*. This preliminary data using a few pituitaries and high dose of SP was followed by a contradictory one, that reported no effect of SP on LH and FSH release by hemipituitaries of OVX rats (Vijayan and McCann, 1979). Using a double-chamber (hypothalamus-pituitary) perfusion system, Ohtsuka and collaborators (1987) were able to show that SP stimulated the *in vitro* release of LH by rat pituitary; this effect was significant only in the presence of hypothalamus, as well as estrogen, and abolished when SP antagonist was also added (Ohtsuka *et al.*, 1987). Kalra and collaborators communicate unpublished data in intact male rats showing that NPK and NPy significantly stimulated LH release *in vitro* from hemipituitaries (Kalra *et al.*, 1992; for review: (Kalra *et al.*, 1991). In cultured porcine gonadotropes, SP was reported to stimulate LH release without affecting intracellular LH

content (Hidalgo-Díaz *et al.*, 1998). It also potentiated GnRH-stimulated LH release and reversed GnRH-induced decrease of gonadotropin LH stores, these effects not being blocked by the use of GnRH-receptor antagonist. A few years later, the same group demonstrated that SP direct effect on pig gonadotropes was extracellular  $Ca^{2+}$ -dependent and did not concern effect on *lhβ* transcript levels (Hidalgo-Díaz *et al.*, 2003).

In the urodele, crested newt *Triturus cristatus*, an indirect measurement of the effect of SP on gonadotropins was performed using a perfusion system combining pituitary and testis/ovary (Gobbetti *et al.*, 2000). Considering the lack of antisera against gonadotropins in this species, the authors measured gonadotropins indirectly through their effects on the secretion of testicular androgens and ovarian progesterone from gonads superfused with the preincubated pituitaries. They concluded that SP downregulated gonadotropin release in both sexes, as pituitaries of both sexes preincubated with SP did not change basal steroid secretion, while pituitaries incubated with medium alone increased their steroid secretion.

In teleost, to date, only one study investigated the direct effects of peptides encoded by *tac1* gene on pituitary hormone expression and release (Hu *et al.*, 2017). Using primary culture of prepubertal carp pituitary cells, Hu and collaborators showed that carp SP and NKA could elevate prolactin (PRL) and somatolactin- $\alpha$  (SL $\alpha$ ) mRNAs and secretion, without any effect on POMC, *fshβ*, *tshβ*, *gp-α*, *gh* and *slβ* expression. For *lhβ*, SP but not NKA could induce a dose-dependent inhibition of mRNA levels (after 24h of treatment) while both induced a dose-dependent stimulation of LH release (after 3h). This induction of *lhβ* release was blocked by the use of TACR1 antagonist, but not TACR2 or TACR3 antagonists, while the inhibition of *lhβ* mRNA by SP was totally abolished in the presence of the same TACR1 antagonist. Moreover, SP was able to partially suppress GnRH-induction of *lhβ* mRNA while co-treatment with TACR1 antagonist enhanced this induction by GnRH.

### 3.3.2.2 *Tac3* peptide (NKB)

To the best of our knowledge, only one *in vitro* study has investigated the potential direct effect of NKB on gonadotropin, by using a gonadotroph cell line (Mijiddorj *et al.*, 2012). The authors reported no effect of NKB on *lhβ* and *fshβ* mRNA expression, even if NKB receptor was detected in this cell line.

In teleosts, most of *in vivo* and *in vitro* (primary cell culture of pituitary cells) data showed increase of gonadotropin release and expression after treatment with TAC3 peptides (*in vivo*, zebrafish: Biran *et al.*, 2012; *in vivo* and *in vitro*, tilapia: Biran *et al.*, 2014; *in vivo*, goldfish: Qi *et al.*, 2015; *in vitro*, striped bass: Zmora *et al.*, 2017; *in vivo*, orange-spotted grouper: Chen *et al.*, 2018). However, some studies reported either no effect (*in vitro*, grass carp: Hu *et al.*, 2014a; *in vivo* and *in vitro*, tilapia: Jin *et al.*, 2016; *in vitro*, orange-spotted grouper: Chen *et al.*, 2018) or inhibitory effect (*in vitro*, tilapia: Jin *et al.*, 2016) of TAC3 peptides on gonadotropins. The effect of the TAC3 peptides varies from species to species, between sexes and during development. For example, zebrafish TAC3a and TAC3RPa induce only LH release when injected to mature females (Biran *et al.*, 2012). Similarly, when injected to goldfish female in mid-vitellogenesis and male in late-spermatogenesis, all three TAC3 peptides (TAC3a, TAC3RPa and TAC3RPb) increase expression of *lhβ* but also *fshβ* in the pituitary and GnRH in the brain (Qi *et al.*, 2015b). In tilapia, when applied *in vitro* to mature female pituitary cells, the two TAC3 (TAC3 and TAC3RP) induce higher increase of FSH and LH release than TAC3RP. Intra peritoneal injections of TAC3 to mature females also increases both FSH and LH plasma levels, while TAC3RP induces only LH release (Biran *et al.*, 2014a). However, if the TAC peptides are applied *in vitro* to juvenile mixed sexes pituitary cells the effect is inhibitory towards expression of *fshβ* and *lhβ* subunits (Jin *et al.*, 2016). In the same study, TAC3RP injected to mature male inhibits the expression of the next gonadotropic genes: *gnrh-ri*, *kiss2* and the same *tac3* gene. TAC3 have been found in LH pituitary cells in tilapia (Biran *et al.*, 2014a) and is likely responsible for direct regulation of *lhβ* expression by endogenous tilapia TAC3 peptides.

### **3.3.3. At the peripheral level**

#### **3.3.3.1. Uterus**

Genes encoding tachykinin peptides (*tac1*, *tac3* and *tac4*) and receptors (*tacr1*, *tacr2* and *tacr3*) were all expressed in the uterus of mouse (Cintado *et al.*, 2001; Pintado *et al.*, 2003; Patak *et al.*, 2005), rat (*tac3*: Pinto *et al.*, 2001) and human (Patak *et al.*, 2003; Pinto *et al.*, 2004), and their expression changed during the estrous cycle and during pregnancy (mouse: Patak *et al.*, 2005; rat: Hamlin *et al.*, 2000; Cadenas *et al.*, 2001).

SP, NKA and NKB, when applied to isolated myometrium from non-pregnant women, produced contractions, while TACR2 receptor-selective antagonist abolished the uterotonic effect of NKA agonist (Patak *et al.*, 2003). Tachykinins (SP, NKA and NKB) produce a direct contractile effect on uterine smooth muscle in mouse (Patak *et al.*, 2002; Pennefather *et al.*, 2004), rat: (Fisher and Pennefather, 1997; Cadenas *et al.*, 2001; Cintado *et al.*, 2001), and human (Patak, 2000b; Patak *et al.*, 2003). Human HK-1 is also a uterine stimulant in the human (Pennefather *et al.*, 2006). TACR2 receptor is mediating tachykinin actions on the myometrium of human (Patak and Story, 2000a; Patak *et al.*, 2003; Pennefather *et al.*, 2006), rat (Moodley *et al.*, 1999).

#### **3.3.3.2. Placenta**

In the placenta of pre-eclampsia women, elevated circulating NKB, as well as increased *tac3* expression, are reported as compared to placenta of normal pregnant women (Page *et al.*, 2000; Page *et al.*, 2006; Page, 2010).

#### **3.3.3.3. Gonads and gametes**

Expression of *tac1* and *tac3* was detected in the mammalian ovary, oocytes and granulosa cells (Pintado *et al.*, 2003; Debeljuk and Lasaga, 2006). Isolated cumulus granulosa cells expressed *tac1*, *tac3* and *tac4* (Pintado *et al.*, 2003). Tachykinins control ovary steroid secretion and may have an ancient role in stimulating oocyte growth as in *Ciona intestinalis* Ci-TACRP enhanced oocyte growth from the vitellogenic stage to the post-vitellogenic stage (Aoyama *et al.*, 2008).

In the male reproductive system (for review: Debeljuk *et al.*, 2003), *tac1*, *tac3* and *tac4* genes are expressed in the human sperm. Tachykinins enhance the sperm motility by TACR1 and TACR2 dependent mechanisms (Pinto *et al.*, 2010). Tachykinins also potentiate contractility dependent mechanisms (Pinto *et al.*, 2010) of the *vas deferens* and of seminal vesicles mediated by TAC1RP (SP) (Candenas *et al.*, 2005). TAC1RP (SP) and TAC1 (NKA) are present in prostate of Guinea pig and rat in low levels, abundant in dog and absent in humans (Candenas *et al.*, 2005), but *tac1*, *tac3* and *tac4* have been detected in human prostate.

## ***4 PhD objectives***

The aim of this PhD is to investigate the presence and the role of brain neuropeptides; such as neurokinin B encoded by *tac3* gene, in the control of reproduction in an endangered species, the European eel, *Anguilla anguilla*.

The eel presents a unique life cycle with a pre-pubertal blockade of sexual maturation as long as the reproductive oceanic migration does not take place. Raising basic knowledge on eel reproductive neuroendocrinology contributes to decipher the regulatory mechanisms of its complex life cycle.

Furthermore, due to its basal phylogenetic position among teleosts, as a representative of Elopomorphs, the eel is also a key-species for addressing molecular and functional evolution of endocrine systems.

The objectives of this PhD are:

1. To identify *in silico* *tac3* gene(s) in *Anguilla* species and predict the sequence and 3D structure of encoded peptides.
2. To analyse the tissue distribution of the *tac3* paralog transcripts in the European eel.
3. To synthesize eel predicted TAC3 peptides and test their biological activity on the expression of pituitary hormones by primary culture of eel pituitary cells.
4. To retrace the evolutionary scenario of *tac3* genes in vertebrate and specially teleosts, by phylogeny and synteny analyses, with a special focus on the impact of whole genome duplication events.
5. To extend *in silico* identification and evolutionary scenario to the other *tac* genes (*tac1* and *tac4*)



### **Material and Methods**

## ***II Materials and Methods***

### ***1 Animals***

European female eels (*Anguilla anguilla*) were at the pre-pubertal “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; N°R-75UPMC-F1-08) according to the protocol approved by Cuvier Ethic Committee France (N°68-027).

### ***2 In silico prediction***

*Tac3* sequences from vertebrate species were retrieved from the Ensembl release 91 ([www.ensembl.org](http://www.ensembl.org)) and NCBI (<https://www.ncbi.nlm.nih.gov/>) databases. Additional blasts were performed using TBLASTN algorithm of the CLC DNA Workbench 6 software (CLC Qiagen, Aarhus, Denmark) in the teleost genomes and multi-organ transcriptomes downloaded from NCBI (<ftp://ftp.ncbi.nlm.nih.gov/genomes/>), Ensembl and Phylofish (<http://phylofish.sigena.org/index.html>, (Pasquier *et al.*, 2016). The sequences of zebrafish *tac3a* (Gene ID: 100320280) and *tac3b* (Gene ID: 569642) (J. Biran *et al.*, 2012) were translated with the EXPASY online tool (<https://web.expasy.org/translate/>) 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: (Henkel *et al.*, 2012a) and nanopore: (Jansen *et al.*, 2017) and on multi-organ transcriptome (ZF-screens B.V (<http://www.zfgenomics.com/sub/eel/>)). In addition, the draft genomes of the Japanese (*Anguilla japonica*) and American (*Anguilla rostrata*) eels (Henkel *et al.*, 2012a; Pavey *et al.*, 2017) were used for prediction of the *tac3* genes using the European eel transcripts as a query.

### ***3 Cloning and sequencing of eel tac3 cDNAs***

The anterior part of the brain including olfactory bulbs, telencephalon and di-/mes-encephalon was dissected and stored in RNA later (Ambion Inc., Austin, TX, USA) at 4°C (24h), then -20°C until extraction. Total RNA was extracted using mechanical homogenization in Trizol Reagent (Invitrogen SARL, Cergy-Pontoise, France), according to manufacturer's instructions. Samples were homogenized by TissueLyser II (QIAGEN, Hilden, Germany) and further treated with deoxyribonuclease I (Roche). RNA quantifications have been performed using a nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA). One microgram of total RNA was reverse-transcribed using SuperScript III First Strand cDNA Synthesis Kit (Invitrogen) and stored at -20°C.

Nested PCRs were performed on anterior brain cDNA using two couples of primers (see Article 1, Table S1). The primers were designed in the 5' and 3' UTRs with Primer 3 online tool (Whitehead Institute/Massachusetts Institute of Technology, Boston, 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 ; 35 cycles of 1 min at 94°C, 1 min at 2°C under the  $T_m$  of the oligonucleotide with the lowest  $T_m$ , 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 MWG Biotech, Ebersberg, Germany).

### ***4 Phylogeny***

18 sarcopterygian, 1 chondrichthyan and 59 actinopterygian TAC3 pre-pro-peptide amino-acid sequences were aligned using MUSCLE and CLUSTAL included in SeaView v. 4.6.1 (Gouy *et al.*, 2010). Alignment was manually adjusted for optimization of key regions such as cleavage sites and signal peptide.

Phylogeny analysis of the TAC3 alignment was achieved using a maximum likelihood method, RAxML black-box (<https://www.phylo.org/>, Stamatakis, 2014), with 1,000 bootstrap replicates and

JTT substitution matrix. The TAC3 pre-pro-peptide sequence from the elasmobranch elephant shark (*Callorhynchus 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 v2.1.

Phylogeny analyses were also performed on some neighbouring genes of *tac3* genomic region : *b4galnt1b* and *c1galt1b*.

Same procedure applied to phylogenetic analysis of TAC1 and TAC4 pre-pro-peptide sequences, where salmonid species were included.

## 5 Synteny

*Tac3* genomic region of a non-teleost actinopterygian, spotted gar, (*Lepisosteus oculatus*) was chosen as reference, using Genomicus PhyloView v91.01 (<http://www.genomicus.biologie.ens.fr/genomicus-91.01/cgi-bin/search.pl>). The genomic regions of eel *tac3a* and *tac3b* were manually analysed with CLC DNA Workbench 6 software (Qiagen Bioinformatics) on *de novo* assembled European eel draft genome (Henkel *et al.*, 2012b; Jansen *et al.*, 2017). The draft genomes of the Japanese (Henkel *et al.*, 2012a) and American (Pavey *et al.*, 2017) eels were also used. *Tac3* neighbouring genes were also identified in other representative teleost genomes (golden arowana, *Sleropages formosus*, osteoglossomorph/osteoglossiform ; Atlantic herring, *Clupea harengus*, clupeomorph/clupeiform; zebrafish, *Danio rerio*, ostariophysii/cypriniform; medaka, *Oryzias latipes* ; tilapia, *Oreochromis niloticus* acanthopterygian/perciform; stickleback, *Gasterosteus acculeatus* acanthopterygian/gasterosteiform; fugu, *Takifugu rubripes* acanthopterygian/tetraodontiform) using CLC DNA Workbench for arowana (assembly accession GCF\_001624265.1) and herring (GFC\_000966335.1) and Genomicus PhyloView for the other species. For each *tac3* neighbouring gene family, when only one gene was annotated in all the above teleost mentioned genomes, blast analyses were performed to search for potential additional paralogs.

## **6 Tissue distribution**

Tissues from ten female silver eels were collected to study the expression of eel *tac3* genes. They were stored in RNA later at 4°C (24h), then -20°C, until extraction. The sampled tissues were brain, pituitary, gill, eye, intestine, liver, spleen, and ovary. The brain was dissected in olfactory bulbs, telencephalon, diencephalon, mesencephalon, *cerebellum* and *medulla oblongata*. Tissue RNA extraction was performed as described in the Cloning section. 500 ng of total RNA were reverse-transcribed using SuperScript III First Strand cDNA Synthesis Kit. Samples were then stored at -20°C until qPCR.

## **7 Prediction and synthesis of eel TAC3 peptides**

SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>) was used for prediction of signal peptide (Petersen *et al.*, 2011), and NeuroPred (<http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py>) for cleavage and amidation sites (Southey *et al.*, 2006).

Predicted eel TAC3 peptides [NKBa (10 aa), NKBRPa (13 aa), NKBb (10 aa) and NKBRPb (13aa)] were synthesized (0.1-mmol scale) by the solid-phase methodology on a Rink amide 4-methylbenzhydrylamine resin (Biochem, Meudon, France) using a 433A peptide synthesizer (Applied Biosystems, Courtaboeuf, France) and the standard procedure, as previously described (30). The synthetic peptides were purified by reversed-phase (RP) HPLC on a 2.2 cm × 25 cm Vydac 218TP1022 C<sub>18</sub> column (Alltech, Templemars, France), using a linear gradient (20–40% over 60 min) of acetonitrile/TFA (99.9:0.1, v/v) in water, at a flow rate of 10 ml/min. Analytical RP-HPLC, performed on a 0.46 cm × 25 cm Vydac 218TP54 C<sub>18</sub> column, showed that the purity of the peptide was greater than 99%. The molecular mass of the peptide was verified by mass spectrometry on a MALDI-TOF Voyager DE-PRO instrument (Applied Biosystems).

## **8 Prediction of the three-dimensional peptide structure of eel TAC3 peptides**

Secondary protein structures of eel TAC3 peptides were modelled using the I-TASSER server, an automated protein-modelling server (Yang *et al.*, 2014). Only models with the C-score between 2 and

-5 were considered. The visualization of the predicted three-dimensional structures was performed using the RasWin Molecular Graphics software v. 2.7.5.2 (<http://www.rasmol.org/>).

## ***9 Pituitary cell culture***

### ***9.1 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 (Montero *et al.*, 1996) and as recently used for the test of eel kisspeptins (Pasquier *et al.*, 2018). Cultures were performed in serum-free culture medium (M199 with Earle's salt, sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml fungizone (Gibco, Illkirch, France) at 18 °C under 3% CO<sub>2</sub> and saturated humidity.

### ***9.2 Treatments***

Human NKB (Sigma) and eel TAC3 peptides stock solutions (10<sup>-4</sup>M) were prepared in NaOH 0.1M and stored at -20°C. Stock solutions were diluted in the culture medium just before the addition to the culture wells. The experiment started at day 0 (24h after cell plating) and five wells (62,500 cells/well) for control and each treatment were used as replicates. Culture medium was changed and peptide treatment solution was added again on days 4 and 7. Culture was stopped at day 10. The effects of the treatment were tested on independent experiments performed on cells from different batches of fish and figures display the results of representative experiments.

### ***9.3 RNA extraction and cDNA synthesis***

Total RNA was directly extracted as previously described (Pasquier *et al.*, 2011, 2018). Briefly, cells were washed with sterile PBS (Gibco, Illkirch, France) and lysed with Cell-to-cDNA™ II Cell Lysis II Buffer (Ambion; 80µl/well). The lysates were digested with RNase-free DNase I (Roche). Eight microliters of RNA solution of each sample were then reversed 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.

## **10 Quantitative real-time PCR (qPCR)**

### **10.1 Primers**

Primers for eel *tac3a* and *tac3b* were designed based on sequences of European eel TAC3 pro-peptides using Primer 3 (Table S1) 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 (Aroua *et al.*, 2007; Pasquier *et al.*, 2011). Primers for eel *lhβ*, *fshβ*, *β subunit of thyroid-stimulating hormone (tshβ)*, *common α subunit of glycoprotein (gpα)*, *type 2 GnRH receptor (gnrh-r2)* and growth hormone (*gh*) have already been described (Table S1). European eel possesses three GnRH receptors (GnRH-R1a, GnRH-R1b and GnRH-R2) (Peñaranda *et al.*, 2013), but *gnrh-r1a* and *gnrh-r1b* expression was below the limit of detection in cultures of pituitary cells (Pasquier *et al.*, 2018), and thus were not assayed in this study.

### **10.2 SYBR green assay**

Quantitative PCR assays were performed using the LightCycler® System (Roche) with SYBR Green I sequence-unspecific detection as previously described (Pasquier *et al.*, 2011, 2018). Briefly, the qPCRs were prepared with 2μl of RNase-free water (Ambion, Texas, USA), 2μl of SYBR Green master mix (Roche), 1μl of each forward and reverse primer (500nM final concentration) and 4μl 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*, *gp α*, *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 fluorescent emission

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  $\beta$ -*actin* mRNA level (cell culture experiments).

## ***11 Statistical analysis***

Data are presented as the mean  $\pm$  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. Normality was tested with Wilk.-Shapiro test using R.



## **Chapter III**

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### **Article 1**

## ***III Article 1***

### ***1 Introduction to article 1***

The first gene to be characterized in the eel genome was the tachykinin 3 because of its reproductive role in mammals and teleosts. Without regard to the presence of the *tac3* gene and diverse TAC3 peptides in other teleost, its presence in the eel was unknown. Therefore, a first *in silico* search for the tachykinin 3 gene in the eel genome was the beginning of this research.

In a practical point of view, the possible role of the tachykinin peptides in the sexual maturation of the eel was still unexplored. Although the effect of the tachykinin peptides in the regulation of gonadotropin release was proven already for mammals and teleosts, there was risk of no reproductive role in the eel.

Thus, the objective of this first study was to reveal the existence of tachykinin genes and peptides in the eel that could take part in the regulation of the sexual maturation. Thanks to the eel genome and bioinformatic tools, the *tac3* gene system was characterised in three eel species. These genes encoded TAC3 peptides that were synthesized and tested *in vitro* in pituitary cells with the objective of discovering any possible role on gonadotropin release. Our qPCR analysis revealed the dual inhibition of *lhβ* and *gnrh-rii* genes in the pituitary cells of silver pre-pubertal eel.

To that end, this chapter is focused on the first characterisation of the tachykinin genes and peptides in the European eel and their implication in the sexual maturation of the pre-pubertal eel.

### ***2 Article 1: Tachykinin-3 genes and peptides characterized in a basal teleost, the European eel: evolutionary perspective and pituitary role***



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

Aurora Campo<sup>1</sup>, Anne-Gaëlle Lafont<sup>1</sup>, Benjamin Lefranc<sup>2</sup>, Jérôme Leprince<sup>2</sup>, Hervé Tostivint<sup>3</sup>, Nédia Kamech<sup>1</sup>, Sylvie Dufour<sup>1</sup> and Karine Rousseau<sup>1\*</sup>

<sup>1</sup>Muséum National d'Histoire Naturelle, Research Unit BOREA (Biology of Aquatic Organisms and Ecosystems), CNRS 7208, IRD 207, Sorbonne Université, Université de Caen Normandie, Université des Antilles, Paris, France, <sup>2</sup>Laboratory of Neuronal and Neuroendocrine Differentiation and Communication, INSERM U1239, Normandy University, Rouen, France, <sup>3</sup>Muséum National d'Histoire Naturelle, UMR7221 CNRS/MNHN Evolution des Régulations Endocriniennes, Paris, France

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### Edited by:

Gustavo M. Somoza,  
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Juan F. Asturiano,  
Universitat Politècnica de  
València, Spain  
Bruno Querat,  
Paris Diderot University, France

### \*Correspondence:

Karine Rousseau  
karine.rousseau@mnhn.fr

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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-I), 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 followed by the loss of one of the four paralogs (*tac2*) (9, 10).

Neurokinin B and its receptor in mammals (TACR3, also named NK3 receptor) [for reviews see Ref. (3, 11)] have been involved in the regulation of the gonadotropic axis, after the discovery that mutations of *tac3* or *tacr3* genes in humans resulted in a hypogonadotropic hypogonadism (12, 13), reversible in adulthood (14). Similarly, although fertile, *tac3* or *tacr3* 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 *tacr3* 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<sup>1</sup> and NCBI<sup>2</sup> 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,<sup>3</sup> Ensembl and Phylofish<sup>4</sup> (39). The sequences of zebrafish *tac3a* (Gene ID: 100320280) and *tac3b* (Gene ID: 569642) (5) were translated with the EXPASY online tool<sup>5</sup> and used as queries. The obtained predicted sequences of the tachykinin pre-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

<sup>1</sup><http://www.ensembl.org/index.html>.

<sup>2</sup><https://www.ncbi.nlm.nih.gov/>.

<sup>3</sup><ftp://ftp.ncbi.nlm.nih.gov/genomes/>.

<sup>4</sup><http://phylofish.sigena.org/index.html>.

<sup>5</sup><https://web.expasy.org/translate/>.



multiorgan transcriptome [ZF-screens B.V.<sup>6</sup>]. In addition, the draft genomes of the Japanese (*Anguilla japonica*) and American (*Anguilla rostrata*) eels (40, 42) were used for prediction of the *tac3* genes using the European eel transcripts as a query. All sequence references are included in Table S1 in Supplementary Material.

## 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). One microgram of total RNA was reverse-transcribed using SuperScript III First Strand cDNA Synthesis Kit (Invitrogen) and stored at -20°C.

Nested PCRs were performed on anterior brain cDNA using two couples of primers (see Table S2 in Supplementary Material). The primers were designed in the 5' and 3' UTRs with Primer 3 online tool (Whitehead Institute/Massachusetts Institute of Technology, Boston, 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; 35 cycles of 1 min at 94°C, 1 min at 2°C under the  $T_m$  of the oligonucleotide with the lowest  $T_m$ , 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 MWG 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 manually 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-box<sup>7</sup> (44), with 1,000 bootstrap replicates and JTT substitution matrix. The TAC3 pre-pro-peptide sequence from the elasmobranchii elephant shark (*Callorhynchus 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

<sup>6</sup>[https://drive.google.com/open?id=1-GfA0fo0xOz7hRc\\_bOYs9ozek2KKd0zK](https://drive.google.com/open?id=1-GfA0fo0xOz7hRc_bOYs9ozek2KKd0zK), <https://drive.google.com/open?id=1YcrkFNy-AIG7WaFsILhs3egHZBvh5VHV>, <https://drive.google.com/open?id=1df2QsMugOfEYfwLq4OieXnosqN3HIDJ>, [https://drive.google.com/open?id=1dWdAfP6GI7piXM9h\\_OPuLITjRrQ7x77](https://drive.google.com/open?id=1dWdAfP6GI7piXM9h_OPuLITjRrQ7x77), [https://drive.google.com/open?id=1QWpkkt\\_WFidqLLNZDBFJaDeFcTMrze8O](https://drive.google.com/open?id=1QWpkkt_WFidqLLNZDBFJaDeFcTMrze8O), [https://drive.google.com/file/d/1onKx-ciZbj\\_PnQgRwAvX2V5jvvgvbxqr/view?usp=sharing](https://drive.google.com/file/d/1onKx-ciZbj_PnQgRwAvX2V5jvvgvbxqr/view?usp=sharing).

<sup>7</sup><https://www.phylo.org/>.

v2.1. Phylogeny analyses were also performed on neighboring genes of *tac3* genomic region: *c1galt1* (core 1 synthase, glyco-protein-N-acetylgalactosamine 3-beta-galactosyltransferase 1) and *b4galnt1* (beta-1,4-N-acetyl-galactosaminyltransferase 1).

## Synteny Analysis

*Tac3* genomic region of a non-teleost actinopterygian, spotted gar (*Lepisosteus oculatus*) was chosen as reference, using Genomicus PhyloView v91.01.<sup>8</sup> The genomic regions of eel *tac3a* and *tac3b* were manually analyzed with CLC Main Workbench 6 software on *de novo* assembled European eel draft genome (40, 41). The draft genomes of the Japanese (40) and American (42) eels were also used. *Tac3* neighboring genes were also identified in other representative teleost genomes (golden arowana, *Scleropages formosus*, osteoglossomorph/osteoglossiform; Atlantic herring, *Clupea harengus*, clupeomorph/clupeiform; zebrafish, *D. rerio*, ostariophysii/cypriniform; medaka, *Oryzias latipes*, acanthopterygii/beloniform; tilapia, *Oreochromis niloticus*, acanthopterygii/perciform; stickleback, *Gasterosteus aculeatus*, acanthopterygii/gasterosteiform; fugu, *Takifugu rubripes* acanthopterygii/tetraodontiform) using CLC DNA Workbench for arowana (assembly accession GCF\_001624265.1) and herring (GFC 000966335.1) and Genomicus PhyloView for the other species. For each *tac3* neighboring gene family, when only one gene was annotated in all the above teleost mentioned genomes, blast analyses were performed to search for potential additional paralogs.

## Tissue Distribution

Tissues from 10 female silver eels were collected to study the expression of eel *tac3* genes. They were stored in RNA later at 4°C (24 h), then at -20°C, until extraction. The sampled tissues were brain, pituitary, gill, eye, intestine, liver, spleen, and ovary. The brain was dissected in olfactory bulbs, telencephalon, diencephalon, mesencephalon, *cerebellum*, and *medulla oblongata*. Tissue RNA extraction was performed as described in the Cloning and Sequencing section. 500 ng of total RNA were reverse-transcribed using SuperScript III First Strand cDNA Synthesis Kit. Samples were then stored at -20°C until qPCR.

## Prediction and Synthesis of Eel TAC3 Peptides

SignalP 4.1<sup>9</sup> was used for prediction of signal peptide (45), and Neuropred<sup>10</sup> for cleavage and amidation sites (46).

Predicted eel TAC3 peptides [NKBa (10 aa), NKB-RPa (13 aa), NKBb (10 aa), and NKB-RPb (13aa)] were synthesized as previously described (47) (Table 1).

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

Secondary structures of eel TAC3 peptides were modeled using the I-TASSER server, an automated protein-modeling server (48). Only models with the C-score between 2 and -5 were considered. The visualization of the predicted three-dimensional structures

<sup>8</sup><http://www.genomicus.biologie.ens.fr/genomicus-91.01/cgi-bin/search.pl>.

<sup>9</sup><http://www.cbs.dtu.dk/services/SignalP/>.

<sup>10</sup><http://stagbeetle.animal.uuic.edu/cgi-bin/neuropred.py>.

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

Gene	Peptide name	Peptide sequence
<i>tac3a</i>	NKBa	DMHDFVGLM-NH <sub>2</sub>
	NKB-RPa	YNGIDYDSFVGLM-NH <sub>2</sub>
<i>tac3b</i>	NKBb	DMDDIFVGLM-NH <sub>2</sub>
	NKB-RPb	YNDIDYDTFVGLM-NH <sub>2</sub>

The predicted sequences are the same for *Anguilla japonica* and *Anguilla rostrata*.

was performed using the RasWin Molecular Graphics software v. 2.7.5.2.<sup>11</sup>

## 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% CO<sub>2</sub> and saturated humidity].

### Treatments

Human NKB (Sigma-Aldrich, Saint-Quentin Fallavier, France) and eel TAC3 peptides stock solutions (10<sup>-4</sup> 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<sup>-12</sup> to 10<sup>-6</sup> M was tested according to previous *in vitro* studies with neuropeptides (47, 50). For synthesized eel NKB and NKB-RP peptides, 10<sup>-7</sup> M was chosen as submaximal response could be observed with 10<sup>-6</sup> 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 fluorescent emission 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

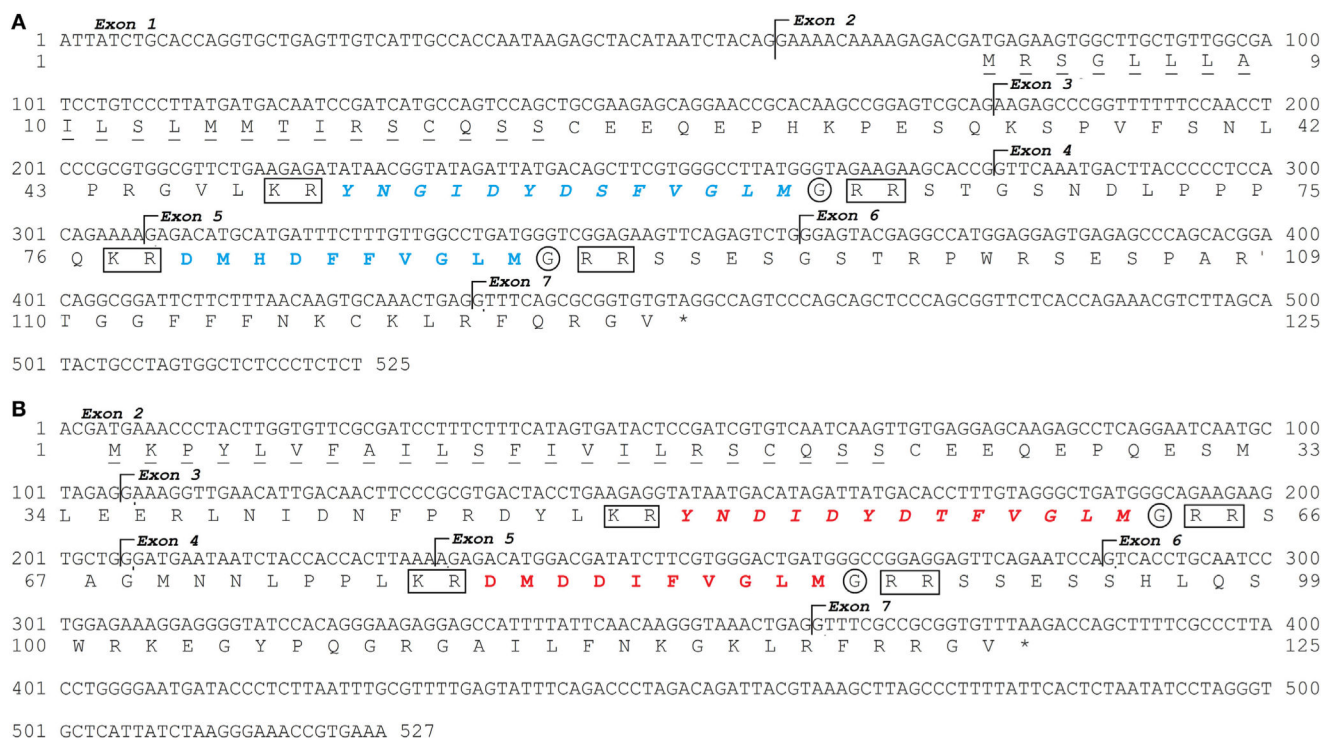
### Characterization of European Eel *tac3* Genes, Transcripts, and Peptides

#### *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

<sup>11</sup><http://www.rasmol.org/>.





**FIGURE 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.

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.

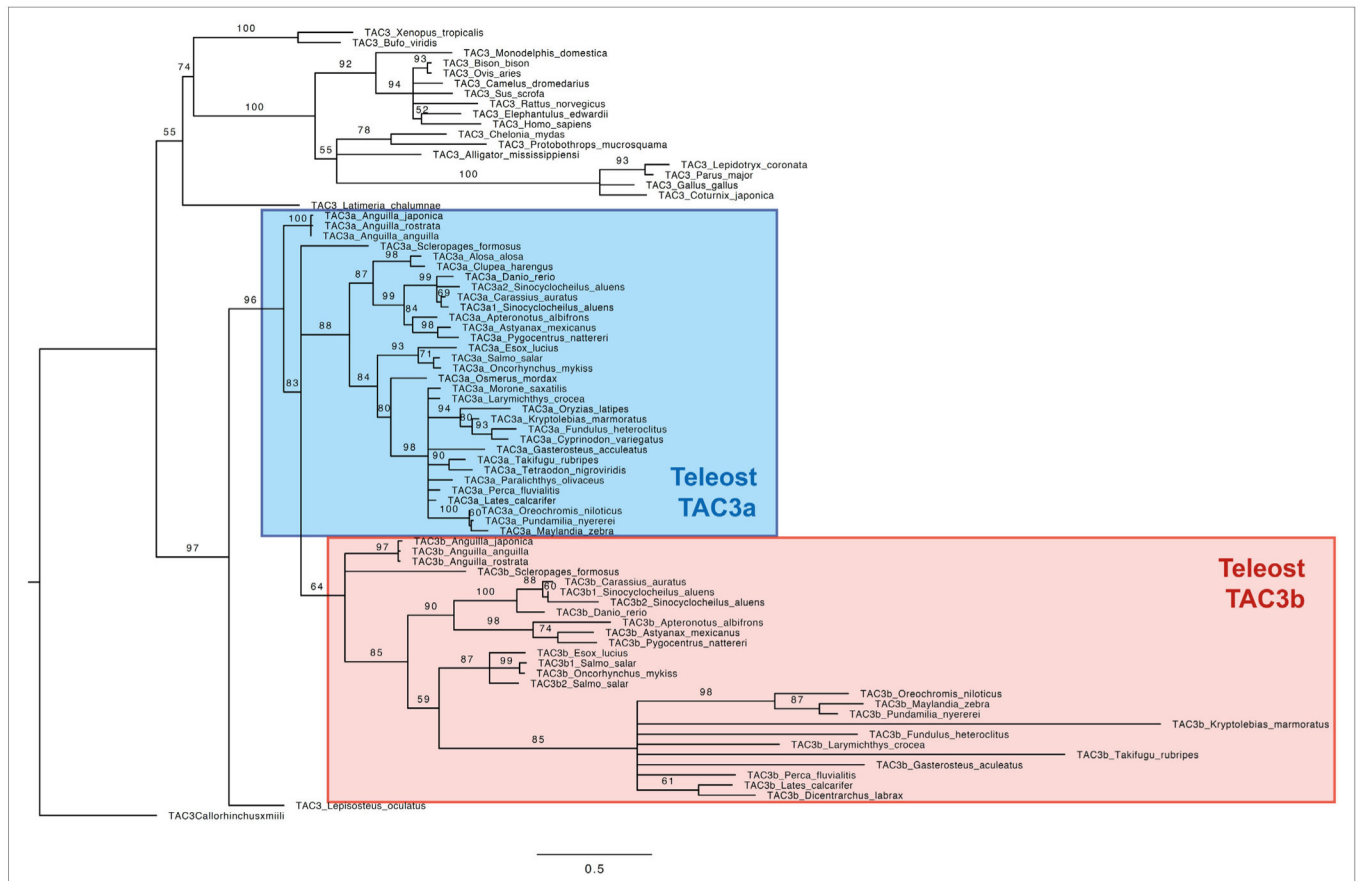
The eel NKBa peptide (10 aa) encoded by *tac3a* gene (**Figure 1**; **Table 1**) has the same sequence as human NKB. This sequence is also conserved in various sarcopterygians (coelacanth, sauropsids, and mammals) as well as in the non-teleost actinopterygian, spotted gar (Figure S1 in Supplementary Material). By contrast, variations in the sequence are observed in all other teleosts studied (Figure S1 in Supplementary Material). The eel NKB-RPa (13 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).

### 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



**FIGURE 2** | Consensus phylogenetic tree of vertebrate TAC3 pre-pro-peptide amino acid sequences. This phylogenetic tree was based on the deduced amino acid sequences of *tac3* (see Table S1 and Figure S1 in Supplementary Material) using the maximum likelihood method with 1,000 bootstrap replicates. The number shown at each branch node indicates the bootstrap value (%); only values and branching above 50% are indicated. The tree was rooted using the TAC3 sequence of the elephant shark *Callorhynchus milii*. The teleost TAC3a and TAC3b sequences are colored in blue and red, respectively.

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.

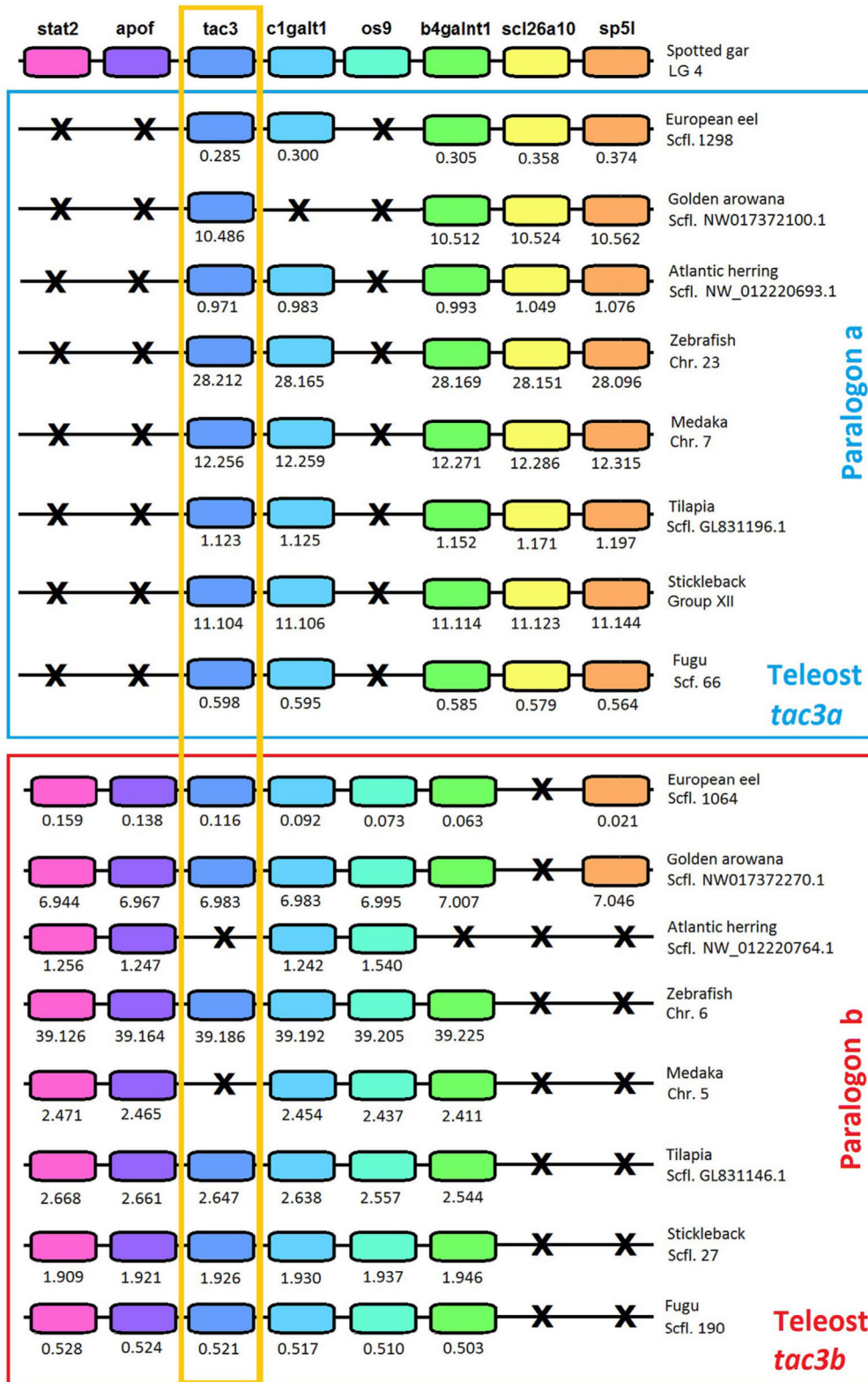
### Synteny Analysis of *tac3* Genomic Region

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 (ostariophysii), 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  $10^9$  bp. The full gene names, reference, and detailed genomic locations are given in Table S3 in Supplementary Material.

and arowana *tac3* sequences to paralogs 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 *tac3a* and *b* paralogs. This phylogeny fully supported the respective assignment of eel and arowana *tac3* sequences to paralogs 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<sup>-7</sup> M, significantly inhibited *lhβ* expression (Figure 5B).

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

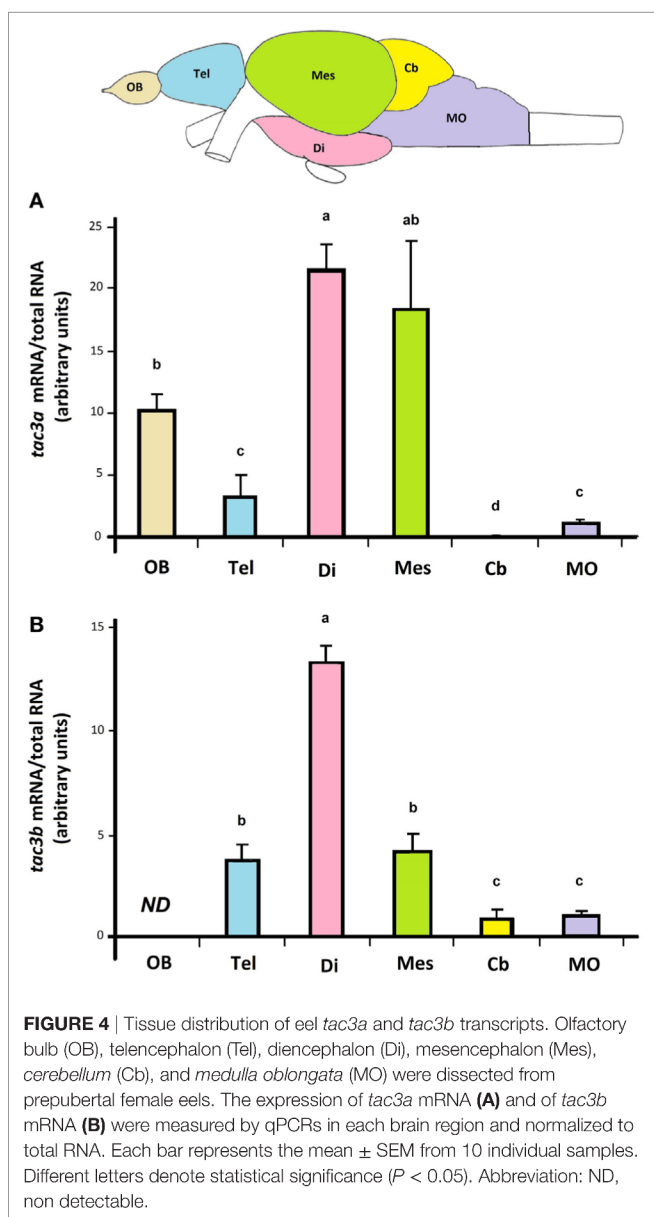
### Prediction of the Three-Dimensional Peptide Structure of Eel TAC3 Peptides

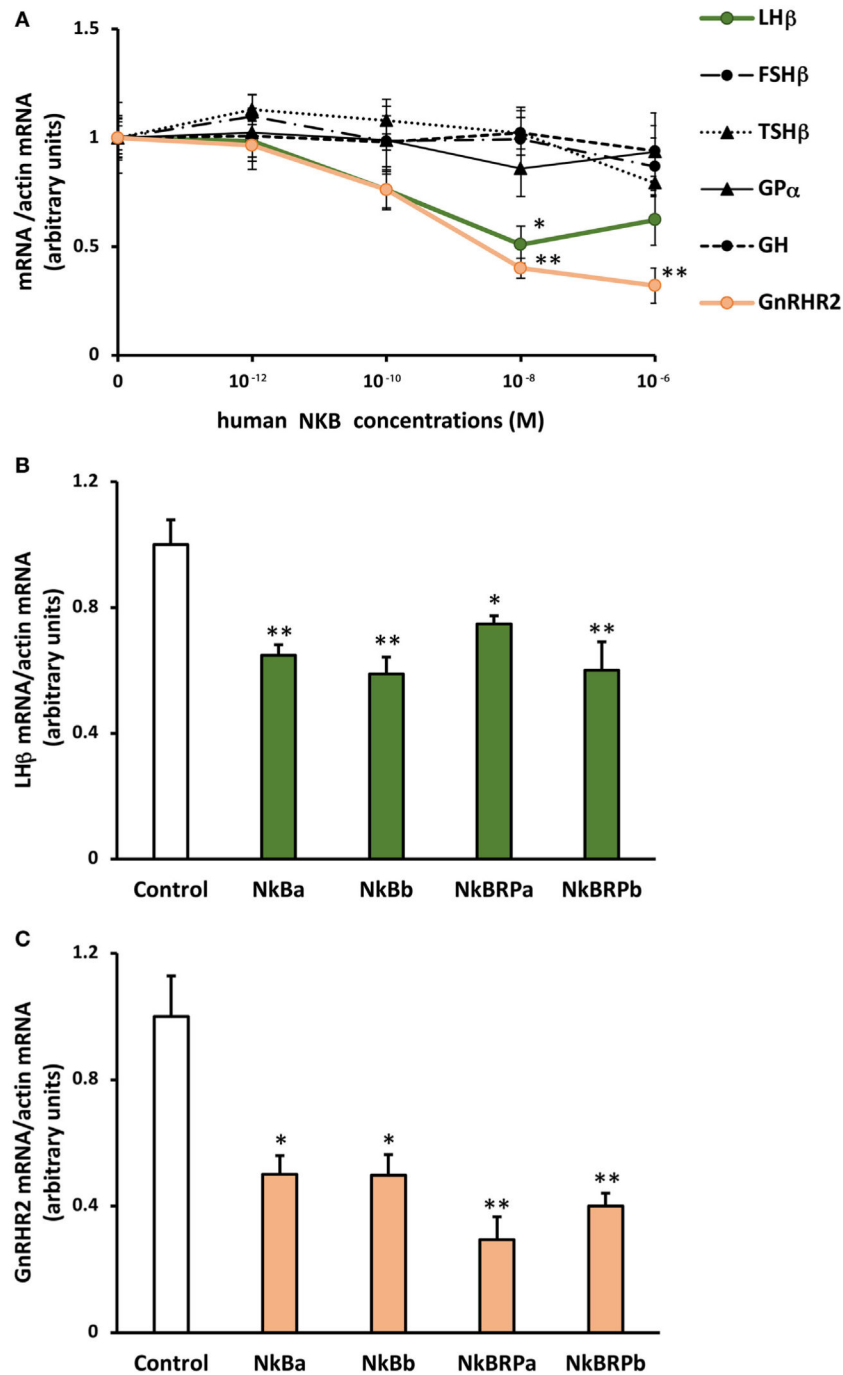
Predicted secondary structures of eel TAC3 peptides were obtained using the I-TASSER server. As described above, eel NKBa peptide sequence is the same as human NKB. Human NKB 3D structure was already reported [PDB ID 1p9f (5)]. For all four eel peptides, the 3D structure was characterized by a single 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





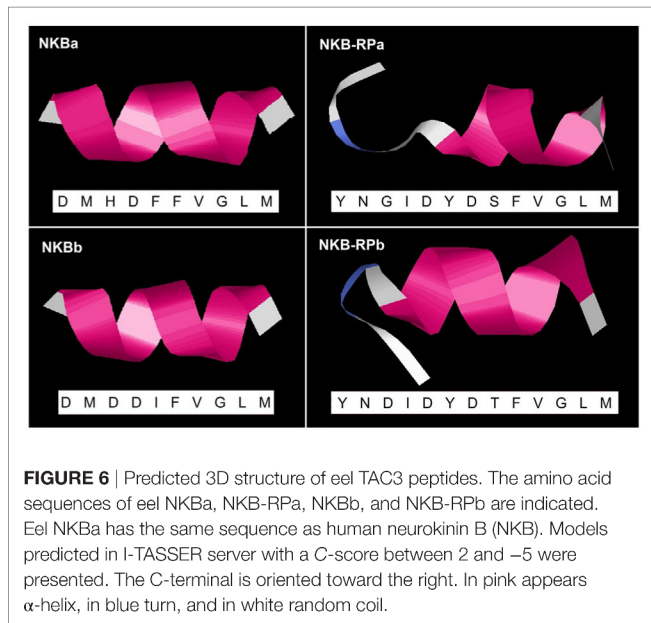
**FIGURE 5 |** Effect of human (A) and eel (B) TAC3 peptides on pituitary hormone and *gnrh* receptor expression by eel pituitary cells. (A) Primary cultures of pituitary cells were treated with various concentrations (10<sup>-12</sup>, 10<sup>-10</sup>, 10<sup>-8</sup>, and 10<sup>-6</sup> M) of human neurokinin B (NKB) for 10 days. Pituitary hormones and receptor mRNA levels were quantified by qPCR. (B,C) Pituitary cells were treated with 10<sup>-7</sup> M of eel TAC3 peptides, NkBa, NkBb, NkB-RPa, and NkB-RPb for 10 days. *lhβ* (B) and *gnrh-r2* (C) mRNA levels were quantified by qPCR. This figure displays the results of representative experiment. Data were normalized against β-actin. Each point represents mean ± SEM from five well replicates. \*P < 0.05 and \*\*P < 0.01 versus controls, t-test or ANOVA.

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





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  $\alpha$ -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 (perciform, 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* mRNAs 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 KNDy 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 *tac3* 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 reviews 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 synthesized eel TAC3 peptides (NKBa, 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 LβT2, 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 ovariectomized 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*, *lhβ*, *fshβ*, *tshβ*, *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 *lhβ* 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 *lhβ* and *gnrh-r2* transcripts in primary cultures of eel pituitary cells. Thus, in the eel, NKB peptides exert a double inhibitory control on gonadotropic function, by decreasing *lhβ* 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-75UPMC-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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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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*Supplementary Material*

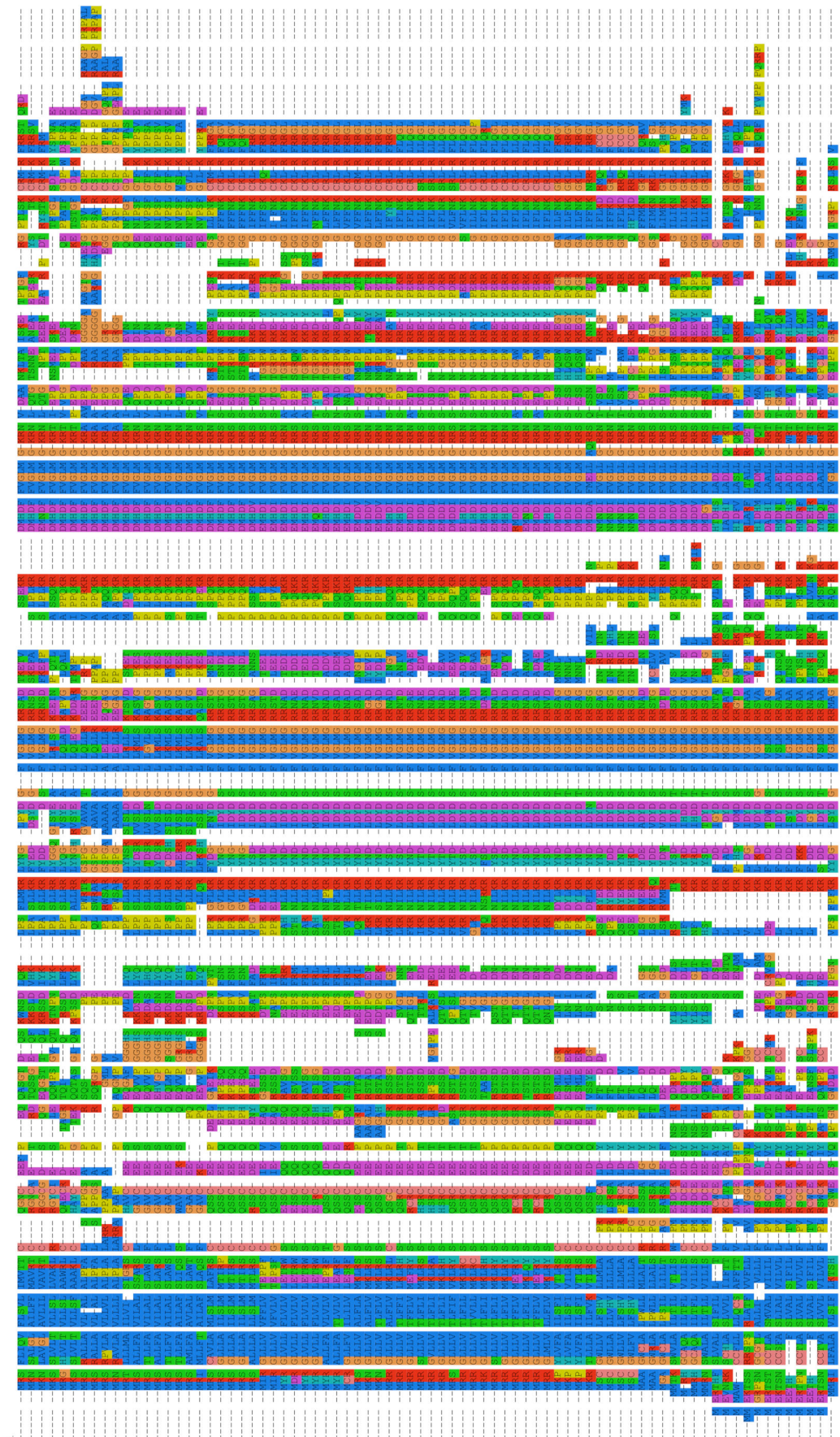
**TACHYKININ-3 GENES AND PEPTIDES CHARACTERIZED IN A  
BASAL TELEOST, THE EUROPEAN EEL: EVOLUTIONARY  
PERSPECTIVE AND PITUITARY ROLE**

Aurora Campo, Anne-Gaëlle Lafont, Benjamin Lefranc, Jérôme Leprince, Hervé Tostivint, Nédia Kamech, Sylvie Dufour and Karine Rousseau

Correspondence: Karine Rousseau: [karine.rousseau@mnhn.fr](mailto:karine.rousseau@mnhn.fr)

## 1. Supplementary Figures

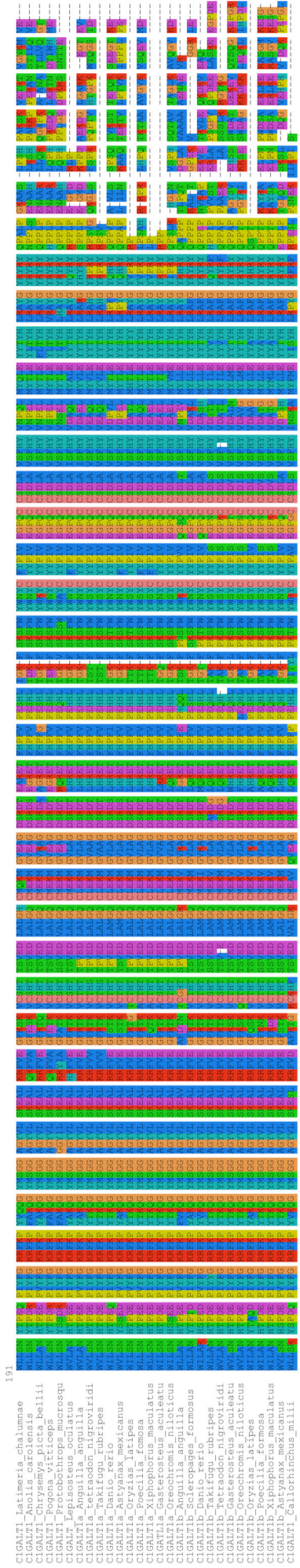
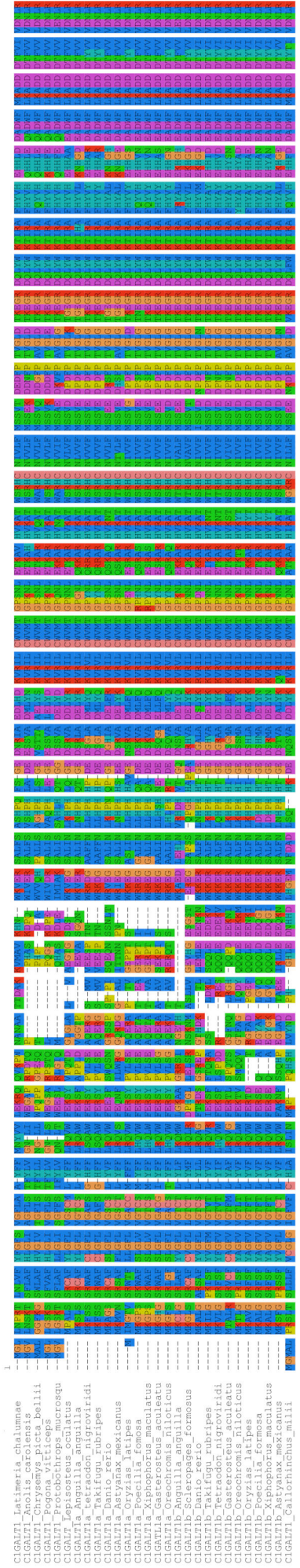
**Supplementary Figure 1.** Alignment of 78 TAC3 pre-pro-peptide sequences used in the phylogeny analysis (Fig 2). The amino-acid residues with similar physico-chemical properties are in the same color.



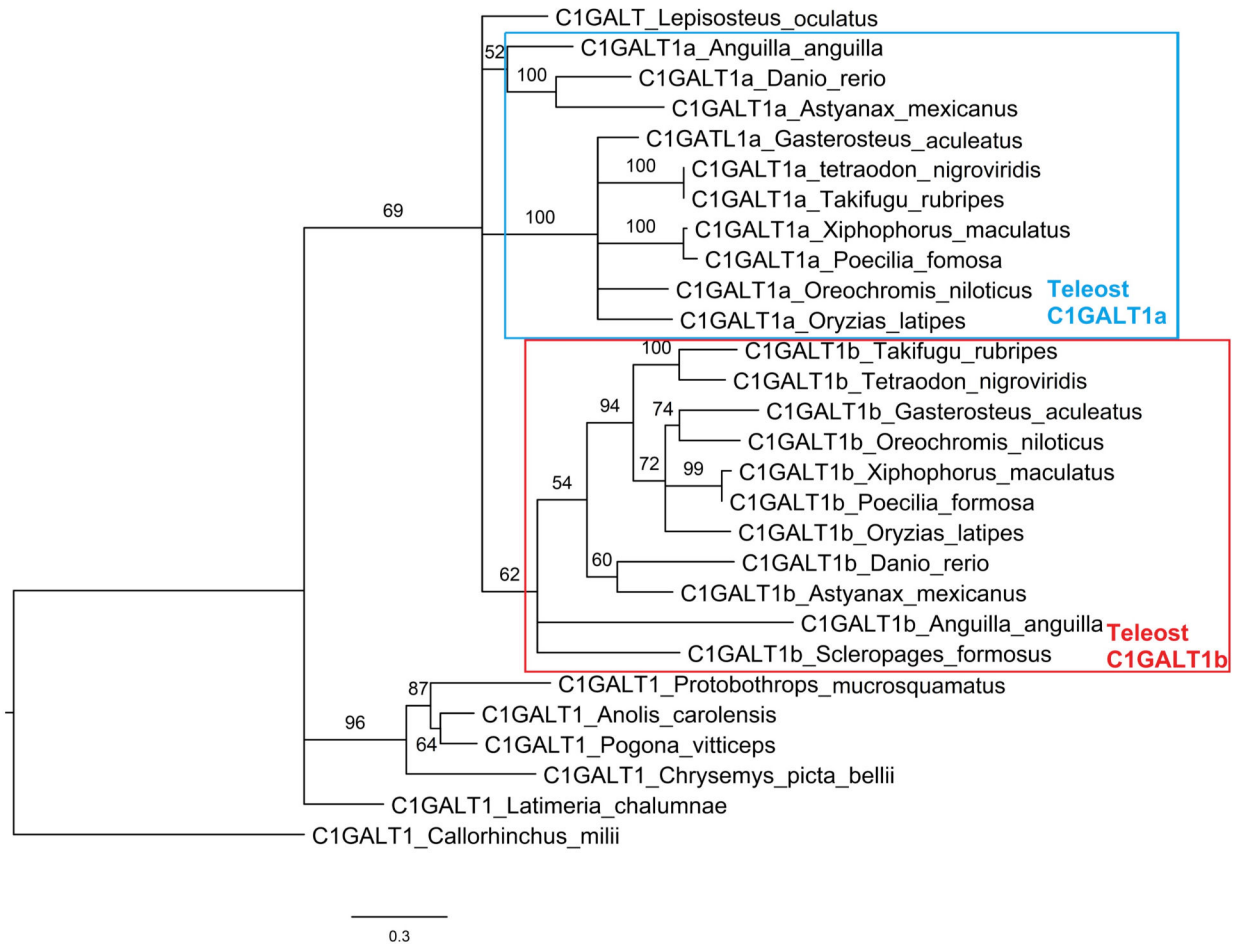
TAC3\_Latimeria\_chalumnae  
 TAC3\_Bufo\_viridis  
 TAC3\_Xenopus\_tropicalis  
 TAC3\_Anguilla\_japonica  
 TAC3\_Alligator\_mississippiensis  
 TAC3\_Cheilonia\_mydas  
 TAC3\_Coturnix\_japonica  
 TAC3\_Gallus\_gallus  
 TAC3\_Pepiderocypris\_coronata  
 TAC3\_Monodelphis\_domestica  
 TAC3\_Bison\_bison  
 TAC3\_Camelus\_dromedarius  
 TAC3\_Ovis\_aries  
 TAC3\_Elephantus\_loxodonta  
 TAC3\_Rattus\_norvegicus  
 TAC3\_Homo\_sapiens  
 TAC3\_Reptisorex\_oculatus  
 TAC3\_Anguilla\_japonica  
 TAC3\_Anguilla\_rostrata  
 TAC3a\_Scleropages\_formosus  
 TAC3a\_Aloea\_alosa  
 TAC3a\_Clupes\_harengus  
 TAC3a\_Pandamia\_nyerelei  
 TAC3a\_Danio\_reio  
 TAC3a2\_Sinocyclocheilus\_albens  
 TAC3a\_Astyanax\_mexicanus  
 TAC3a\_Apterontotus\_albifrons  
 TAC3a\_Esox\_lucius  
 TAC3a Oncochinchus\_mykiss  
 TAC3a\_Salmo\_salar  
 TAC3a\_Osmotreron\_dax  
 TAC3a\_Oreochromis\_niloticus  
 TAC3a\_Cyprinodon\_variegatus  
 TAC3a\_Fundulus\_heteroclitus  
 TAC3a\_Kryptolebias\_marmoratus  
 TAC3a\_Taki\_fugu\_rubripes  
 TAC3a\_Tetraodon\_nigroviridis  
 TAC3a\_Gasterosteus\_aculeatus  
 TAC3a\_Larymichthys\_crocea  
 TAC3a\_Wayu\_gaetzeri  
 TAC3a\_Perca\_fluviatilis  
 TAC3a\_Pundamilia\_nyerelei  
 TAC3a\_Morone\_saxatilis  
 TAC3b\_Anguilla\_japonica  
 TAC3b\_Anguilla\_rostrata  
 TAC3b\_Scleropages\_formosus  
 TAC3b\_Garassius\_auratus  
 TAC3b\_Danio\_reio  
 TAC3b\_Esox\_lucius  
 TAC3b2\_Sinocyclocheilus\_albens  
 TAC3b\_Pygocentrus\_nattereri  
 TAC3b\_Apterontotus\_albifrons  
 TAC3b\_Salmo\_salar\_mykiss  
 TAC3b2\_Salmo\_salar  
 TAC3b\_Oreochromis\_niloticus  
 TAC3b\_Fundulus\_heteroclitus  
 TAC3b\_Taki\_fugu\_rubripes  
 TAC3b\_Larymichthys\_crocea  
 TAC3b\_Lates\_gaetarifer  
 TAC3b\_Saylandia\_rubripes  
 TAC3b\_Pundamilia\_nyerelei  
 TAC3b\_Dicentrarchus\_labrax  
 TAC3\_Callorhynchus\_mullii

**Supplementary Figure 2.** Alignment of 28 C1GALT1 amino-acid sequences used in the phylogeny analysis (Fig S3). The amino-acid residues with similar physico-chemical properties are in the same color.



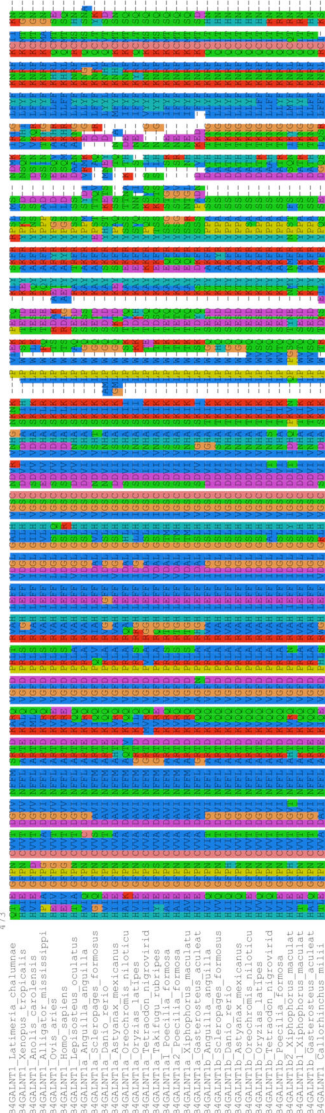
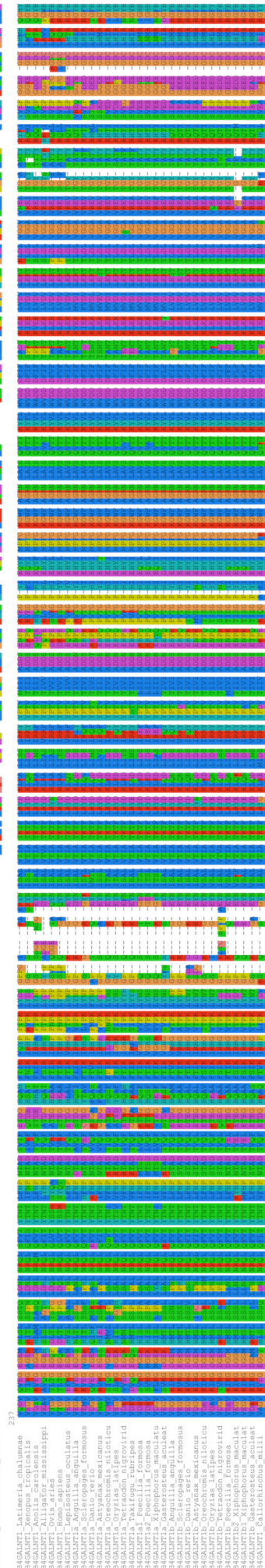
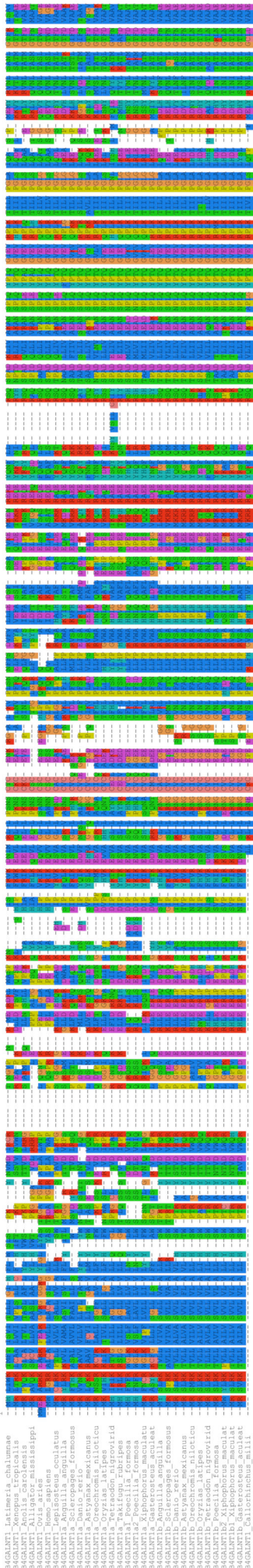


**Supplementary Figure 3.** Consensus phylogenetic tree of vertebrate C1GALT1. This phylogenetic tree was based on the amino-acid sequences of C1GALT1 (Table S4) using the Maximum Likelihood method with 1,000 bootstrap replicates. The number shown at each branch node indicates the bootstrap value (%); only values above 50% are indicated. The tree was rooted using the C1GALT1 sequence of the elephant shark *Callorhinchus milii*. The sequences corresponding to paralogon “a” and “b” are framed in blue and red, respectively.



**Supplementary Figure 4.** Alignment of 30 B4GALTN1 amino-acid sequences used in the phylogenetic (Fig S5). The amino-acid residues with similar physico-chemical are in the same color.









TAC3	<i>Gallus gallus</i>	XM_015300406.1	NCBI
TAC3	<i>Lepidothryx coronata</i>	XM_017836603.1	NCBI
TAC3	<i>Parus major</i>	XM_015615656.1	NCBI
TAC3	<i>Monodelphis domestica</i>	XM_007506244.2	NCBI
TAC3	<i>Bison bison</i>	XM_010839305.1	NCBI
TAC3	<i>Camelus dromedarius</i>	XM_010990978.1	NCBI
TAC3	<i>Ovis aries</i>	XM_015094789.1	NCBI
TAC3	<i>Sus scrofa</i>	NM_001007196.1	Li et al. 2005
TAC3	<i>Elephantulus edwardii</i>	XM_006897674.1	NCBI
TAC3	<i>Rattus norvegicus</i>	NM_019162.2	Ruiz Pino et al. 2015
TAC3	<i>Homo sapiens</i>	KJ892241.1	Yang et al. 2011
TAC3	<i>Lepisosteus oculatus</i>	XM_015344463.1	NCBI
TAC3a	<i>Anguilla anguilla</i>	cloned	Present article
TAC3b	<i>Anguilla anguilla</i>	cloned	Present article
TAC3a	<i>Anguilla japonica</i>	predicted	Present article
TAC3b	<i>Anguilla japonica</i>	predicted	Present article
TAC3a	<i>Anguilla rostrata</i>	predicted	Present article
TAC3b	<i>Anguilla rostrata</i>	predicted	Present article
TAC3a	<i>Scleropages formosus</i>	predicted	Present article
TAC3b	<i>Scleropages formosus</i>	predicted	Present article
TAC3a	<i>Alosa alosa</i>	predicted	Present article
TAC3a	<i>Clupea harengus</i>	XM_012824242.1	NCBI
TAC3a	<i>Carassius auratus</i>	KF177342.1	Qi et al. 2015 (ref 32)
TAC3b	<i>Carassius auratus</i>	KF177343.1	Qi et al. 2015 (ref 32)
TAC3a	<i>Danio rerio</i>	JN392856.1	Biran et al. 2012 (ref 5)
TAC3b	<i>Danio rerio</i>	JN392857.1	Biran et al. 2012 (ref 5)
TAC3a1	<i>Sinocyclocheilus anshuiensis</i>	XM_016478326.1	NCBI
TAC3a2	<i>Sinocyclocheilus anshuiensis</i>	XM_016495847.1	NCBI
TAC3b1	<i>Sinocyclocheilus anshuiensis</i>	predicted	Present article
TAC3b2	<i>Sinocyclocheilus anshuiensis</i>	predicted	Present article
TAC3a	<i>Astianax mexicanus</i>	XM_007246760.3	NCBI
TAC3b	<i>Astianax mexicanus</i>	predicted	Present article
TAC3a	<i>Pygocentrus nattereri</i>	XM_017706258.1	NCBI
TAC3b	<i>Pygocentrus nattereri</i>	predicted	Present article
TAC3a	<i>Apteronotus albifrons</i>	predicted	Present article
TAC3b	<i>Apteronotus albifrons</i>	predicted	Present article
TAC3a	<i>Esox lucius</i>	XM_010875105.3	NCBI
TAC3b	<i>Esox lucius</i>	XM_010881641.2	NCBI
TAC3a	<i>Oncorhynchus mykiss</i>	XM_021565858.1	NCBI
TAC3b	<i>Oncorhynchus mykiss</i>	predicted	Present article
TAC3a	<i>Salmo salar</i>	BK008102.1	Biran et al. 2012 (ref 5)
TAC3b1	<i>Salmo salar</i>	BK008103.1	Biran et al. 2012 (ref 5)
TAC3b2	<i>Salmo salar</i>	predicted	Present article

TAC3a	<i>Osmerus mordax</i>	BK008111.1	Biran et al. 2012 (ref 5)
TAC3a	<i>Paralichthys olivaceus</i>	XM_020089538.1	NCBI
TAC3a	<i>Oreochromis niloticus</i>	KF471673.1	Biran et al. 2014 (ref 31)
TAC3b	<i>Oreochromis niloticus</i>	predicted	Present article
TAC3a	<i>Cyprinodon variegatus</i>	XM_015377892.1	NCBI
TAC3a	<i>Fundulus heteroclitus</i>	predicted	Present article
TAC3b	<i>Fundulus heteroclitus</i>	predicted	Present article
TAC3a	<i>Kryptolebias marmoratus</i>	XM_017430900.1	NCBI
TAC3b	<i>Kryptolebias marmoratus</i>	predicted	Present article
TAC3a	<i>Oryzas latipes</i>	NM_001278903.1	Biran et al. 2012 (ref 5)
TAC3a	<i>Takifugu rubripes</i>	XM_011621888.1	NCBI
TAC3b	<i>Takifugu rubripes</i>	predicted	Present article
TAC3a	<i>Tetraodon nigroviridis</i>	CR713079	Jaillon et al. 2004
TAC3a	<i>Gasterosteus acculeatus</i>	predicted	Present article
TAC3b	<i>Gasterosteus acculeatus</i>	predicted	Present article
TAC3a	<i>Larymichthys crocea</i>	XM_010747871.2	NCBI
TAC3b	<i>Larymichthys crocea</i>	predicted	Present article
TAC3a	<i>Lates calcarifer</i>	XM_018666039.1	NCBI
TAC3b	<i>Lates calcarifer</i>	XR_001960617.1	NCBI
TAC3a	<i>Maylandia zebra</i>	predicted	Present article
TAC3b	<i>Maylandia zebra</i>	predicted	Present article
TAC3a	<i>Perca fluviatilis</i>	predicted	Present article
TAC3b	<i>Perca fluviatilis</i>	predicted	Present article
TAC3a	<i>Pundamilia nyererei</i>	XM_005732601.1	NCBI
TAC3b	<i>Pundamilia nyererei</i>	predicted	Present article
TAC3b	<i>Dicentrarchus labrax</i>	BK008116.1	Biran et al. 2012 (ref 5)
TAC3a	<i>Morone saxatilis</i>	KT361626.1	Zmora et al. 2017 (ref 34)
TAC3	<i>Callorhinchus milii</i>	XM_007885087.1	NCBI

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Ruiz Pino F et al. Effects and interactions of tachykinins and dynorphin on FSH and LH secretion in developing adult rats. *Endocrinology* (2015) 156:576-588.

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Jaillon O et al. Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature* (2004) 431:946-957.

**Supplementary Table 2.** List of PCR and qPCR primers. Gene names are indicated by the same acronym used in the text.

Primer ID	PCR type	5' - 3' Sequence	T <sub>m</sub> (C)	Amplicon size (bp)	References
TAC3a F	qPCR	<i>ATCCGATCATGCCAGTCCAG</i>	59.4	140	Present article
TAC3a R	qPCR	<i>ATAAGGCCACGAAGCTGTC</i>	59.4		
TAC3b F	qPCR	<i>CATTGACAACCTCCCGCTG</i>	59.4	190	Present article
TAC3b R	qPCR	<i>TCTCCAGGATTGCAGGTGAC</i>	59.4		
LHβ F	qPCR	<i>TCACCTCCTTGTCTGCTG</i>	57.43	149	Aroua et al. 2007 ( <a href="#">ref 53</a> )
LHβ R	qPCR	<i>TAGCTTGGGTCCTTGGTGATG</i>	60.83		
FSHβ F	qPCR	<i>TCTCGCCAACATCTCCATC</i>	58.09	100	Aroua et al. 2007 ( <a href="#">ref 53</a> )
FSHβ R	qPCR	<i>AGAATCCTGGGTGAAGCACA</i>	59.09		
GnRH-R2 F	qPCR	<i>TCACCTTCTCCTGCCTCTTC</i>	59.4	105	Peñaranda et al. 2014 ( <a href="#">ref 55</a> )
GnRH-R2 R	qPCR	<i>TTGGAAGATGCCTTCCCTTT</i>	55.3		
β-actin F	qPCR	<i>AGTATTTGCGCTCGGGTG</i>	58.25	226	Aroua et al. 2007 ( <a href="#">ref 53</a> )
β-actin R	qPCR	<i>CAGCCTTCCTCCTGGGT</i>	58.6		
GPα F	qPCR	<i>TGCCGACTCCAGGAGAATAA</i>	59.21	184	Aroua et al. 2007 ( <a href="#">ref 53</a> )
GPα R	qPCR	<i>TGTTATCCAGCCTTGTACC</i>	57.01		
TSHβ F	qPCR	<i>GCTTCTGCTATTCCCTGGAC</i>	59.4	295	Maugars et al. 2014 ( <a href="#">ref 54</a> )
TSHβ R	qPCR	<i>AGGGTCTGGATGTAGTTGCTC</i>	59.8		
GH F	qPCR	<i>ACCGTCACCTACATCCTTCAT</i>	57.9	183	Aroua et al. 2007 ( <a href="#">ref 53</a> )
GH R	qPCR	<i>AAATCGGATGGGTACTTGCTG</i>	57.9		
TAC3a UTR F	PCR	<i>AACTCTCCGCTTCCACATCA</i>	57.3	559	Present article
TAC3a UTR R	PCR	<i>CTGGAACCAAAAAGAAGGAGCA</i>	57.9		
TAC3b UTR F	PCR	<i>AACTCAACGGCTGACAGAGA</i>	57.3	429	Present article
TAC3b UTR R	PCR	<i>CCCAAGTTTCACGGTTTCCA</i>	57.3		
TAC3aNES F	PCR	<i>TCTCCGCTTCCACATCAGAG</i>	59.4	330	Present article
TAC3aNES R	PCR	<i>AGAAGGAGCAAACCTAGGCAG</i>	57.3		
TAC3bNES F	PCR	<i>CGGCTGACAGAGAGACACAA</i>	59.4	521	Present article
TAC3bNES R	PCR	<i>CATTCCAGTAAAGGGCGAAAAG</i>	58.4		
TAC3aORF F	PCR	<i>GGCGATCCTGTCCCTTATGA</i>	59.4	508	Present article
TAC3aORF R	PCR	<i>CTACACGCCGCGCTGAAA</i>	58.2		
TAC3bORF F	PCR	<i>TACTTGGTGTTCGCGATCCT</i>	57.3	320	Present article
TAC3bORF R	PCR	<i>TTAAACACCGCGGCGAAAC</i>	56.7		

**Supplementary Table 3.** Start and end positions of genes used for the synteny analysis (Fig 3). Species are separated as follows: Spotted gar (A), European eel (B), Golden arowana (C), Atlantic herring (D), Zebrafish (E), Medaka (F), Tilapia (G), Stickleback (H), Fugu (I).

**A- Spotted gar**

Gene name	Chromosome	Strand	Start	End	Name
<i>APOF</i>	4	-	18360102	18360599	<i>predicted</i>
<i>b4galnt1</i>	4	+	18,451,123	18,469,616	ENSLOCG00000007549
<i>c1galt1</i>	4	-	18,413,115	18,417,342	ENSLOCG00000007520
<i>os9</i>	4	-	18,424,244	18,440,368	ENSLOCG00000007533
<i>slc26a10</i>	4	-	18,475,722	18,488,327	ENSLOCG00000007565
<i>sp5l</i>	4	+	18,530,676	18,538,905	ENSLOCG00000007577
<i>stat2</i>	4	-	18,320,440	18,348,846	ENSLOCG00000007484
<i>TAC3</i>	4	+	18,400,010	18,406,027	ENSLOCG00000007508

**B European eel**

Gene name	Scaffold	Scaffold Illumina	Strand	Start	End	Name
	<i>NOT</i>		<i>NOT</i>			
<i>APOFa</i>	<i>FOUND</i>	<i>NOT FOUND</i>	<i>FOUND</i>	<i>NOT FOUND</i>	<i>NOT FOUND</i>	<i>NOT FOUND</i>
		7234 + 41297 + 393457 + 371373				
<i>APOFb</i>	1064	+ 215787	+	138304	141388	<i>predicted</i>
<i>b4galnt1a</i>	1298		926 +	305580	332147	DAA_B4GN1.4.4
<i>b4galnt1b</i>	1064		997 +	63122	43899	DAA_B4GN1.1.4
<i>c1galt1a</i>	1298		926 -	300490	294895	g8752
<i>c1galt1b</i>	1064		997 +	92158	100195	DAA_LOC101071001.1.1
	<i>NOT</i>		<i>NOT</i>			
<i>os9a</i>	<i>FOUND</i>	<i>NOT FOUND</i>	<i>FOUND</i>	<i>NOT FOUND</i>	<i>NOT FOUND</i>	<i>NOT FOUND</i>
<i>os9b</i>	1064		997 +	73850	91790	g9139
<i>slc26a10a</i>	1298		926 -	358535	336898	g8754
	<i>NOT</i>		<i>NOT</i>			
<i>slc26a10b</i>	<i>FOUND</i>	<i>NOT FOUND</i>	<i>FOUND</i>	<i>NOT FOUND</i>	<i>NOT FOUND</i>	<i>NOT FOUND</i>
<i>sp5la</i>	1298		279 +	374224	376850	g4163
<i>sp5lb</i>	1064	259 + 264931	-	21439	20372	<i>predicted</i>
	<i>NOT</i>		<i>NOT</i>			
<i>stat2a</i>	<i>FOUND</i>	<i>NOT FOUND</i>	<i>FOUND</i>	<i>NOT FOUND</i>	<i>NOT FOUND</i>	<i>NOT FOUND</i>
<i>stat2b</i>	1064	11124+contig1975	-	159207	144007	g25275
<i>TAC3a</i>	1298		926 +	285648	293166	<i>predicted</i>
<i>TAC3b</i>	1064		997 -	116556	102959	<i>predicted</i>

**B- Golden arowana**

Gene name	Scaffold	Strand	Start	End	Name
<i>APOFa</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>APOFb</i>	NW_017372270.1	-	6967277	6964631	XP_018581191.1
<i>b4galnt1a</i>	NW_017372100.1	+	10512021	10522571	XM_018760130.1
<i>b4galnt1b</i>	NW_017372270.1	+	7007769	7028122	XM_018726028.1
<i>c1galt1a</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>c1galt1b</i>	NW_017372270.1	-	6983706	6995592	XP_018580957.1
<i>os9a</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>os9b</i>	NW_017372270.1	+	6995931	7005853	XP_018580723.1
<i>slc26a10a</i>	NW_017372100.1	-	10524752	10534501	XP_018615803.1
<i>slc26a10b</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>sp5la</i>	NW_017372100.1	+	10562179	10562428	XP_01861590.1
<i>sp5lb</i>	NW_017372270.1	+	7046475	7049846	XP_018580928.1
<i>stat2a</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>stat2b</i>	NW_017372270.1	-	6944862	6962876	XP_018581189.1
<i>TAC3a</i>	NW_017372100.1	+	10486419	10490938	predicted
<i>TAC3b</i>	NW_017372270.1	+	6983657	6988623	predicted

### C- Atlantic herring

Gene name	Scaffold	Strand	Start	End	Name
		NOT	NOT	NOT	
<i>APOFa</i>	NOT FOUND	FOUND	FOUND	FOUND	NOT FOUND
<i>APOFb</i>	NW_012220764	+	1247562	1251616	XM_012824856.1
<i>b4galnt1a</i>	NW_012220693	+	993981	1036038	XM_012824238.1
		NOT	NOT	NOT	
<i>b4galnt1b</i>	NOT FOUND	FOUND	FOUND	FOUND	NOT FOUND
<i>c1galt1a</i>	NW_012220693	-	983589	976247	XM_012824193.1
<i>c1galt1b</i>	NW_012220764	+	1242824	1245850	XM_012824880.1
		NOT	NOT	NOT	
<i>os9a</i>	NOT FOUND	FOUND	FOUND	FOUND	NOT FOUND
<i>os9b</i>	NW_012220764	+	1540408	1572781	XM_012824950.1
<i>slc26a10a</i>	NW_012220693	-	1049705	1043574	XM_012824197.1
		NOT	NOT	NOT	
<i>slc26a10b</i>	NOT FOUND	FOUND	FOUND	FOUND	NOT FOUND
<i>sp5la</i>	NW_012220693		1076150	1079368	XM_012824198.1
		NOT	NOT	NOT	
<i>sp5lb</i>	NOT FOUND	FOUND	FOUND	FOUND	NOT FOUND
		NOT	NOT	NOT	
<i>stat2a</i>	NOT FOUND	FOUND	FOUND	FOUND	NOT FOUND
<i>stat2b</i>	NW_012220764	+	1256363	1271064	XM_012824856.1
<i>TAC3a</i>	NW_012220693	+	971516	972842	XM_012824242.1
		NOT	NOT	NOT	
<i>TAC3b</i>	NOT FOUND	FOUND	FOUND	FOUND	NOT FOUND

**D- Zebrafish**

Gene name	Scaffold	Strand	Start	End	Name
<i>APOFa</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>APOFb</i>	6	-	39,164,758	39,170,196	ENSDARG00000090980
<i>b4galnt1a</i>	23	-	28,169,082	28,197,226	ENSDARG00000061520
<i>b4galnt1b</i>	6	+	39,225,062	39,255,315	ENSDARG00000077352
<i>c1galt1a</i>	23	+	28,165,542	28,212,580	ENSDARG00000055585
<i>c1galt1b</i>	6	-	39,192,866	39,201,534	ENSDARG00000055561
<i>os9a</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>os9b</i>	6	-	39,205,149	39,221,073	ENSDARG00000020301
<i>slc26a10a</i>	23	+	28,151,412	28,164,568	ENSDARG00000078800
<i>slc26a10b</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>sp5la</i>	23	-	28,096,555	28,099,402	ENSDARG00000010124
<i>sp5lb</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>stat2a</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>stat2b</i>	6	-	39,126,008	39,162,886	ENSDARG00000031647
<i>TAC3a</i>	23	-	28,212,948	28,214,878	ENSDARG00000093089
<i>TAC3b</i>	6	+	39,186,700	39,190,121	ENSDARG00000097596

## E- Medaka

Gene name	Scaffold	Strand	Start	End	Name
<i>APOFa</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>APOFb</i>		5 +	2,465,799	2,468,618	ENSORLG00000001646
<i>b4galnt1a</i>		7 +	12,271,869	12,282,550	XM_020704642.1
<i>b4galnt1b</i>		5 -	2,411,270	2,421,401	ENSORLG00000001618
<i>c1galt1a</i>		7 -	12,259,498	12,263,422	ENSORLG00000007498
<i>c1galt1b</i>		5 +	2,454,606	2,459,872	ENSORLG00000001643
<i>os9a</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>os9b</i>		5 +	2,437,952	2,449,467	ENSORLG00000001640
<i>slc26a10a</i>		7 -	12,286,683	12,291,323	ENSORLG00000007519
<i>slc26a10b</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>sp5la</i>		7 +	12,315,360	12,317,000	ENSORLG00000007526
<i>sp5lb</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>stat2a</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>stat2b</i>		5 +	2,471,673	2,476,751	ENSORLG00000001651
<i>TAC3a</i>		7 +	12,256,959	12,258,340	ENSORLG00000007492
<i>TAC3b</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND



**F- Tilapia**

Gene name	Scaffold	Strand	Start	End	Name
<i>APOFa</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>APOFb</i>	GL831146.1	+	2,661,668	2,662,945	ENSONIG00000021171
<i>b4galnt1a</i>	GL831196.1	+	1,152,564	1,166,785	ENSONIG00000012109
<i>b4galnt1b</i>	GL831146.1	-	2,544,566	2,553,108	ENSONIG00000019085
<i>c1galt1a</i>	GL831196.1	-	1,125,478	1,130,612	ENSONIG00000012108
<i>c1galt1b</i>	GL831146.1	+	2,638,855	2,643,692	ENSONIG00000019092
<i>os9a</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>os9b</i>	GL831146.1	+	2,557,277	2,568,899	ENSONIG00000019088
<i>slc26a10a</i>	GL831190.1	-	1,171,402	1,178,570	ENSONIG00000012113
<i>slc26a10b</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>sp5la</i>	GL831196.1	+	1,197,578	1,200,437	ENSONIG00000012117
<i>sp5lb</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>stat2a</i>	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND	NOT FOUND
<i>stat2b</i>	GL831146.1	+	2,668,069	2,677,496	ENSONIG00000019094
<i>TAC3a</i>	GL831196.1	-	1,123,378	1,124,183	NM_001311342.1
<i>TAC3b</i>	GL831146.1	-	2,647,978	2,653,087	predicted

## G- Stickleback

Gene name	Scaffold	Strand	Start	End	Name
	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	
<i>APOFa</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>NOT FOUND</i>
<i>APOFb</i>		27 -	1,921,736	1,923,064	<i>ENSGACG00000000709</i>
<i>b4galnt1a</i>	<i>XII</i>	+	11,114,611	11,120,117	<i>Predicted</i>
<i>b4galnt1b</i>		27 +	1,946,114	1,954,478	<i>ENSGACG00000000713</i>
<i>c1galt1a</i>	<i>XII</i>	-	11,106,920	11,109,223	<i>ENSGACG000000008969</i>
<i>c1galt1b</i>		27 +	1,930,982	1,935,286	<i>ENSGACG00000000710</i>
	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	
<i>os9a</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>NOT FOUND</i>
<i>os9b</i>		27 -	1,937,087	1,945,286	<i>ENSGACG00000000711</i>
<i>slc26a10a</i>	<i>XII</i>	-	11,123,251	11,128,216	<i>ENSGACG000000008976</i>
	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	
<i>slc26a10b</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>NOT FOUND</i>
<i>sp5la</i>	<i>XII</i>	+	11,144,529	11,145,968	<i>ENSGACG000000008991</i>
	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	
<i>spl5b</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>NOT FOUND</i>
	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	
<i>stat2a</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>NOT FOUND</i>
<i>stat2b</i>		27 -	1,909,074	1,917,435	<i>ENSGACG00000000707</i>
<i>TAC3a</i>	<i>XII</i>	+	11,104,847	11,105,875	<i>Predicted</i>
<i>TAC3b</i>		27 +	1,926,921	1,928,465	<i>Predicted</i>

**H- Fugu**

Gene name	Scaffold	Strand	Start	End	Name
	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	
<i>APOFa</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>NOT FOUND</i>
<i>APOFb</i>	190	+	524,324	526,594	<i>XM_003973211.2</i>
<i>b4galnt1a</i>	66	-	585,666	592,675	<i>Predicted</i>
<i>b4galnt1b</i>	190	-	503,710	506,328	<i>ENSTRUG00000012842</i>
<i>c1galt1a</i>	66	+	595,206	597,481	<i>ENSTRUG00000013143</i>
<i>c1galt1b</i>	190	+	517,503	519,125	<i>ENSTRUG00000013063</i>
	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	
<i>os9a</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>NOT FOUND</i>
<i>os9b</i>	190	+	510,886	515,522	<i>ENSTRUG00000012933</i>
<i>slc26a10a</i>	66	+	579,350	583,361	<i>ENSTRUG00000012857</i>
	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	
<i>slc26a10b</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>NOT FOUND</i>
<i>sp5la</i>	66	-	0,564,330	565,723	<i>ENSTRUG00000012835</i>
	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	
<i>sp5lb</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>NOT FOUND</i>
	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	<i>NOT</i>	
<i>stat2a</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>FOUND</i>	<i>NOT FOUND</i>
<i>stat2b</i>	190	+	528,290	532,939	<i>ENSTRUG00000013090</i>
<i>TAC3a</i>	66	-	598402	599852	<i>predicted</i>
<i>TAC3b</i>	190	+	521828	520608	<i>predicted</i>

**Supplementary Table 4.** Name and accession number of the C1GALT1 amino-acid sequences used in the alignment (Fig S2) and phylogeny analysis (Fig S3).

Pre-pro-peptide	Species	Accession number	Annotation provider
C1GALT1	<i>Latimeria chalumnae</i>	ENSLACG00000018257	Ensemble
C1GALT1	<i>Anolis carolensis</i>	ENSACAG00000006384	Ensemble
C1GALT1	<i>Pogona vitticeps</i>	XP_020633885.1	NCBI
	<i>Protobothrops</i>		
C1GALT1	<i>mucrosquamatus</i>	XP_015672638.1	NCBI
C1GALT1	<i>Chrysemys picta belli</i>	XP_023956690.1	NCBI
C1GALT1	<i>Lepisosteus oculatus</i>	ENSLOC00000007520	Ensemble
C1GALT1a	<i>Anguilla anguilla</i>	a_1298 DAA_C1GTA.2.3	Phylofish
C1GALT1b	<i>Anguilla anguilla</i>	b_1064 DAA_LOC101071001.1.1	Phylofish
C1GALT1b	<i>Scleropages formosus</i>	COB_C1GTB.1.1	Phylofish
C1GALT1a	<i>Danio rerio</i>	ENSDARG00000055585	Ensemble
C1GALT1b	<i>Danio rerio</i>	ENSDARG00000055561	Ensemble
C1GALT1a	<i>Astyanax mexicanus</i>	ENSAMXG00000013071	Ensemble
C1GALT1b	<i>Astyanax mexicanus</i>	ENSAMXG00000001374	Ensemble
C1GALT1a	<i>Oreochromis niloticus</i>	ENSONIG00000012108	Ensemble
C1GALT1b	<i>Oreochromis niloticus</i>	ENSONIG00000019092	Ensemble
C1GALT1a	<i>Oryzias latipes</i>	ENSORLG00000007498	Ensemble
C1GALT1b	<i>Oryzias latipes</i>	ENSORLG00000001643	Ensemble
C1GALT1a	<i>Takifugu rubripes</i>	ENSTRUG00000013143	Ensemble
C1GALT1b	<i>Takifugu rubripes</i>	ENSTRUG00000013063	Ensemble
C1GALT1a	<i>Tetraodon nigroviridis</i>	ENSTNIG00000015077	Ensemble
C1GALT1b	<i>Tetraodon nigroviridis</i>	ENSTNIG00000014583	Ensemble
C1GALT1a	<i>Gasterosteus aculeatus</i>	ENSGACG00000008969	Ensemble
C1GALT1b	<i>Gasterosteus aculeatus</i>	ENSGACG00000000710	Ensemble
C1GALT1a	<i>Poecilia formosa</i>	ENSPFOG00000008682	Ensemble
C1GALT1b	<i>Poecilia formosa</i>	ENSPFOG00000011251	Ensemble
C1GALT1a	<i>Xiphophorus maculatus</i>	ENSXMAG00000004968	Ensemble
C1GALT1b	<i>Xiphophorus maculatus</i>	ENSXMAG00000002052	Ensemble
C1GALT1a	<i>Gadus morhua</i>	ENSGMOG00000001538	Ensemble
C1GALT1b	<i>Gadus morhua</i>	ENSGMOG00000007524	Ensemble
C1GALT1	<i>Callorhinchus milii</i>	XP_007902959.1	NCBI

**Supplementary Table 5.** Name and accession number of the B4GALNT1 amino-acid sequences used in the alignment (Fig S4) and phylogeny analysis (Fig S5).

Pre-pro-peptide	Species	Accession number	Annotation provider
B4GALNT1	<i>Latimeria chalumnae</i>	ENSLACG00000018149	Ensembl
B4GALNT1	<i>Xenopus tropicalis</i>	NP_001120168.1	NCBI
B4GALNT1	<i>Anolis carolensis</i>	ENSACAG00000007194	Ensembl
B4GALNT1	<i>Alligator mississippiensis</i>	XP_014464072.1	NCBI
B4GALNT1	<i>Ovis aries</i>	ENSOARP00000006086	Ensembl
B4GALNT1	<i>Homo sapiens</i>	ENSG00000135454	Ensembl
B4GALNT1	<i>Lepisosteus oculatus</i>	ENSLOCG00000007549	Ensembl
B4GALNT1a	<i>Anguilla anguilla</i>	DAA_B4GN1.4.4	Phylofish
B4GALNT1b	<i>Anguilla anguilla</i>	DAA_B4GN1.1.4	Phylofish
B4GALNT1a	<i>Scleropages formosus</i>	XM_018760130.1	NCBI
B4GALNT1b	<i>Scleropages formosus</i>	XM_018726028.1	NCBI
B4GALNT1a	<i>Danio rerio</i>	ENSDARG000000061520	Ensembl
B4GALNT1b	<i>Danio rerio</i>	ENSDARG000000077352	Ensembl
B4GALNT1a	<i>Astyanax mexicanus</i>	ENSAMXG00000001403	Ensembl
B4GALNT1b	<i>Astyanax mexicanus</i>	TAM_LOC793635.2.2	Phylofish
B4GALNT1a	<i>Oreochromis niloticus</i>	ENSONIG00000012109	Ensembl
B4GALNT1b	<i>Oreochromis niloticus</i>	ENSONIG00000019085	Ensembl
B4GALNT1a	<i>Oryzias latipes</i>	XM_020704642.1	NCBI
B4GALNT1b	<i>Oryzias latipes</i>	ENSORLGG00000001618	Ensembl
B4GALNT1a	<i>Takifugu rubripes</i>	Predicted	
B4GALNT1a	<i>Tetraodon nigroviridis</i>	Predicted	
B4GALNT1b1	<i>Tetraodon nigroviridis</i>	ENSTNIG00000014585	Ensembl
B4GALNT1a	<i>Gasterosteus aculeatus</i>	Predicted	
B4GALNT1b	<i>Gasterosteus aculeatus</i>	ENSGACG00000000713	Ensembl
B4GALNT1a1	<i>Poecilia formosa</i>	ENSPFOG00000008471	Ensembl
B4GALNT1a2	<i>Poecilia formosa</i>	ENSPFOG00000000900	Ensembl
B4GALNT1b1	<i>Poecilia formosa</i>	ENSPFOG00000011391	Ensembl
B4GALNT1a	<i>Xiphophorus maculatus</i>	XM_023344035.1	NCBI
B4GALNT1b1	<i>Xiphophorus maculatus</i>	ENSXMAG00000002029	Ensembl
B4GALNT1b2	<i>Xiphophorus maculatus</i>	ENSXMAG0000001838	Ensembl
B4GALNT1	<i>Callorhynchus milii</i>	XM_007909947.1	NCBI

## **Chapter IV**

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### **Article 2**

## ***IV Article 2***

### ***1 Introduction to article 2***

The bioinformatic analysis of the eel species and other teleosts for the search of the tachykinin 3 genes revealed an impact of the teleost whole genome duplication or 3R in the teleost tachykinins. In this scenario, we decided to widen the perspective of the tachykinin genes and peptides in teleost.

Our *in silico* analysis discovered that the tachykinin genes *tac1* and *tac4* in the eel species, *Anguilla anguilla*, have been affected by the whole teleost duplication. Together with the *tac3* gene, the European eel has conserved up to 6 tachykinin genes. Each of these genes encodes two peptides with preserved cleavage and amidation sites, hence likely active peptides. Consequently, the eel has potentially 12 tachykinin peptides with hidden pharmacologic potential.

These genes have also been duplicated and preserved in most of the studied teleosts, likely after the teleost whole genome duplication. We could report only independent losses and most of the clades preserve also the two encoded peptides in each gene.

To better understanding the impact of genome duplications in the tachykinin system, we analysed the duplications occurred after the salmonids whole genome duplication event, or 4R. Our results indicate that the tachykinin peptides in salmonids were also preserved in duplicate after their specific genome duplication and a total of 24 tachykinin peptides were found in most of the salmonid species studied.

In conclusion, the impact of the teleost whole genome duplication in the tachykinin system extended the possibilities for tachykinin peptides.

### ***2 Article 2: Tachykinin-1 and 4 genes and peptides in a basal teleost, Anguilla anguilla: Identification and phylogeny analysis***

*In preparation*

**Tachykinin-1 and 4 genes and peptides in a basal teleost,  
*Anguilla anguilla*: identification and phylogeny analysis**

Aurora Campo<sup>1</sup> et al.

Research Unit BOREA, Biology of Aquatic Organisms and Ecosystems, Muséum National d'Histoire Naturelle, CNRS, Sorbonne Université, UCN, UA, Paris, France

*Keywords:* tachykinin-1, tachykinin-4, phylogeny, whole genome duplication, teleost, *Anguilla*



## ***INTRODUCTION***

Tachykinins (TK or TAC) are members of a large family of peptides, mainly produced by brain and gut in mammals. Their C-terminal end was characterized by a motif of five residues (FxGLM) and an  $\alpha$ -amidation. The most known tachykinin peptides are neurokinin A (NKA), substance P (SP) and neurokinin B (NKB). While the *tac1* gene encodes NKA and SP, NKB is coded by the *tac3* gene (also named *tac2* in rodents) (for reviews: Severini et al., 2002; Pennefather et al., 2004; Hu et al., 2014b). In 2000, a third gene, *tac4* gene was identified in mammals and shown to encode other tachykinins: hemokinin-1 (HK-1) and endokinins (EKs) (for reviews: Patacchini et al., 2004; Page et al., 2009). *Tac 1, 3* and *4* paralogous genes are likely resulting from the whole genome duplication events that occurred in ancestral vertebrates (1R and 2R, for “first and second rounds on whole genome duplication”), while the fourth paralog would have been lost early after the 2R.

We recently characterized in the European eel two *tac3* paralogous genes (*tac3a* and *tac3b*) resulting from the additional, teleost-specific, whole genome duplication (TSWGD or 3R for “third round of whole genome duplication”). Each paralog encodes two peptides, thus leading to four TAC3 peptides in the eel: TAC3a (or NKBa) with a sequence identical to that of human NKB, TAC3b (or NKBb), TAC3-RPa (or NKB-RPa) and TAC3-RPb (or NKB-RPb) (Campo et al., 2018). Few studies addressed the other tachykinins in teleosts. Zhou et al (2012) reported the presence of two *tac4* genes (*tac4a* and *b*) in zebrafish and medaka, but the presence of a single *tac1* gene in these species.

The present study aimed at identifying European eel *tac1* and *tac4* genes by *in silico* data mining, and at predicting the sequences of the respective encoded peptides. We also searched for *tac1* and *tac4* genes in various other species and performed phylogeny analyses of *tac1* and *tac4* with a special focus on teleosts.

## ***MATERIALS AND METHODS***

### ***In silico prediction of *tac1* and *tac4* genes***

Tac1 and tac4 sequences from vertebrate species were retrieved from the Ensembl release 91 ([www.ensembl.org](http://www.ensembl.org)) and NCBI (<https://www.ncbi.nlm.nih.gov/>) databases. Additional blasts were performed using TBLASTN algorithm of the CLC Main Workbench 6 software (Qiagen Bioinformatics) in the teleost genomes and multi-organ transcriptomes downloaded from NCBI (<ftp://ftp.ncbi.nlm.nih.gov/genomes/>), Ensembl and Phylofish (<http://phylofish.sigenae.org/index.html>; Pasquier et al., 2016). The sequences of zebrafish *tac1*, *tac4a* and *tac4b* (Zhou et al., 2012) were translated with the EXPASY online tool (<https://web.expasy.org/translate/>) and used as queries. The obtained predicted sequences of the tachykinin pre-propeptides were added to the query list for a new multiblast in the next species.

For the European eel, blast analyses were performed on both available draft genomes (Illumina, Henkel et al., 2012a and nanopore, Janson et al., 2017) and on multi-organ transcriptome (ZF-screens B.V <http://www.zfgenomics.com/sub/eel>). In addition, the draft genomes of the Japanese (*Anguilla japonica*) (Henkel et al., 2012b) and American (*Anguilla rostrata*) eels (Pavey et al., 2017) were used.

### ***Prediction of eel TAC1 and TAC4 peptides***

SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>) was used for prediction of signal peptide (Petersen et al., 2011), and Neuropred (<http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py>) for cleavage and amidation sites (Southey et al., 2006).

## ***Phylogeny analysis***

Chondrichthyan, sarcopterygian, and actinopterygian TAC1 or TAC4 pre-pro-peptide amino-acid sequences were aligned using CLUSTAL included in SeaView v. 4.6. Alignments were manually adjusted for optimization of key regions such as cleavage sites and signal peptide.

Phylogeny analyses of the TAC1 or TAC4 alignments were achieved using a maximum likelihood method, RAxML black-box (<https://www.phylo.org/>), with 1,000 bootstrap replicates and JTT substitution matrix. The TAC1 or TAC4 pre-propeptide sequences from the elasmobranch elephant shark (*Callorhinchus milii*) were chosen as outgroup, respectively. The resulting phylogenetic trees were displayed using Figtree v1.4.3. Nodes were collapsed for bootstrapping values below 30% using Mesquite v2.1.

## ***RESULTS***

### ***Identification of European eel *tac1* and *tac4* genes and encoded peptides***

Two *tac1* genes and two *tac4* genes were identified in the European eel genome. Predicted CDS and corresponding pre-pro-peptide sequences are shown in Figures 1 and 2.

Each TAC1 and TAC4 pre-pro-peptide sequence encoded two putative peptides, TAC and TAC-Related Peptide (TAC-RP) (Figures 1 and 2). These peptides were delimited by cleavage sequences at both ends. The C-terminal end of each peptide sequence showed a glycine before the cleavage site, which indicates an amidation site.

Predicted sequences of European eel peptides TAC1a, TAC1-RPa, TAC1b, TAC1-RPb, TAC4a, TAC4-RPa, TAC4b, TAC4-RPb are given in Table 1, as well as the corresponding human tachykinin peptides, TAC1 (also called Neurokinin A, NKA), TAC1-RP (also called Substance, SP), TAC4 (also called Endokinin, EKC/D), TAC4-RP (also called Hemokinin, HK, and Endokinin EKA/B). The eight eel predicted peptides are identical in the three eel species studied, European eel,

*Anguilla anguilla*, American eel, *Anguilla rostrata*, Japanese eel, *Anguilla japonica*, and represent novel peptide sequences (Table 1; Figure S1 and S2).

### ***Phylogeny analysis of TAC1***

Based on an alignment of 90 TAC1 pre-propeptide amino-acid sequences (Figure S1), and assuming the elephant shark *Callorhynchus milii* sequence as outgroup, a phylogenetic tree was generated using the Maximum Likelihood method (Figure 3).

As shown in Figure 3, the TAC1 pre-propeptide sequences of osteichthyans clustered into two well-supported clades: sarcopterygians and actinopterygians. The coelacanth TAC1 pre-propeptide sequence branched at the base of the sarcopterygian clade, in agreement with its phylogenetic position. Similarly, the TAC1 pre-propeptide sequence of the spotted gar *Lepisosteus oculatus*, a holostean species, branched at the base of the actinopterygian TAC1 clade.

In most teleosts, including zebrafish and medaka, two *tac1* genes (a and b) were found. One clade grouped all teleosts TAC1a pre-propeptide sequences, including the TAC1 previously reported in zebrafish *Danio rerio* and some other teleosts (Zhou *et al.*, 2012). TAC1a sequences from basal teleosts, Elopomorphs (eels) and Osteoglossomorph, branched at the base of the teleost TAC1a clade, in agreement with their basal phylogenetic position. Another well-supported clade encompassed eel TAC1b sequence and clupeccephala TAC1b sequences. Long branches suggested important TAC1b pre-propeptide sequence divergence in clupeccephala. Osteoglossomorph TAC1b pre-propeptide sequences branched at the base of all teleost TAC1a and b pre-propeptide sequences. *Tac1b* gene could not be retrieved in the genome of some teleost species, such as stickleback *Gasterosteus aculeatus* and fugu *Takifugu rubripes*. In contrast, up to four *tac1* genes were identified in salmonids, with two *tac1a* and two *tac1b* paralogs in several *Onchorynchus* species and in *Salvelinus alpinus*.

## ***Phylogeny analysis of TAC4***

Based on an alignment of 64 TAC4 pre-propeptide amino-acid sequences (Figure S2), and assuming the elephant shark *Callorhynchus milii* sequence as outgroup, a phylogenetic tree was generated using the Maximum Likelihood method (Figure 4).

The osteichthyans TAC4 pre-propeptide sequences clustered into two well-supported clades: sarcopterygians and actinopterygians. The coelacanth *Latimeria chalumnae* TAC4 pre-propeptide sequence branched at the base of the sarcopterygian clade, in agreement with its phylogenetic position. Long branches suggested important TAC4 pre-propeptide sequence divergence in amniotes.

The holostean (spotted gar) TAC4 pre-propeptide sequence branched at the base of the actinopterygian TAC4 clade, in agreement with its phylogenetic position. Two *tac4* genes were found in various teleost species, including in zebrafish *Danio rerio* and medaka *Oryzias latipes* as previously reported (Zhou et al., 2012). Teleost sequences split into two clades, TAC4a and TAC4b. Only a single *tac4* gene was retrieved in osteoglossomorph genomes, branching at the base of the teleost TAC4b clade. Up to four *tac4* genes were identified in salmonids, with two *tac4a* and two *tac4b* paralogs in several *Onchorynchus* species.

## ***DISCUSSION***

### **Two *tac1* genes and four TAC1 peptides in the eel**

Our results showed that the European eel, a basal teleost representative of Elopomorphs, as well as other eel species, the American eel and the Japanese eel, possess two *tac1* paralogous genes, each of them encoding two peptides, TAC1a or b and TAC-Related-Peptide (TAC-RPa or b). This organization of the precursor coding for two peptides TAC and TAC-RP is a conserved feature for tachykinins, as exemplified by human TAC1 pre-propeptide including NKA (TAC1) and SP (TAC1-

RP). The sequence of eel TAC1 peptides, TAC1a and b, TAC1-RPa and b, differed from human NKA and SP are not common to any other studied species. Therefore, they represent novel sequences.

To our knowledge, this is the first demonstration of two *tac1* genes in teleosts, since the previous study by Zhou et al. (2012) reported the presence of a single *tac1* gene in the studied teleosts, namely zebrafish *Danio rerio*, medaka *Oryzias latipes*, Atlantic salmon *Salmo salar* and rainbow smelt *Osmerus mordax*.

Based on our finding of two *tac1* genes in a basal teleost, the eel, we further searched and retrieved duplicated *tac1* genes in many other teleost species (Figure 3 and Figure S1) including the above mentioned, differently from the previous report (Zhou *et al.*, 2012). However, we identified only a single *tac1* gene (*tac1a*) in the available genomes of some other teleosts, fugu and stickleback.

### ***Origin and fate of duplicated *tac1a* and *b* paralogs in teleosts***

In order to investigate the origin and relationships of the teleost *tac1a* and *b* paralogs, we performed a phylogenetic analysis of TAC1 pre-propeptide sequences. A single *tac1* gene was identified in chondrichtian as well as in sarcopterygian species such as coelacanth or human. We also retrieved a single *tac1* gene in the non-teleost actinopterygian, a holostean species, the spotted gar. In contrast, we found that two *tac1* genes are present in the eel as well as in most other teleost species investigated. This suggests that the duplicated *tac1* paralogs in teleosts may result from the teleost-specific whole genome duplication (TSWGD or 3R for “Third Whole genome duplication”).

Our phylogeny analysis supported this hypothesis. The spotted gar TAC1 pre-propeptide sequence branched at the base of all TAC1a and TAC1b teleost sequences, indicating a common actinopterygian ancestral *tac1* gene for teleost duplicated *tac1a* and *tac1b* genes. Furthermore, teleost sequences split into two clades, one clade encompassing all TAC1a pre-propeptide sequences and the other one all TAC1b pre-propeptide sequences, with the exception of osteoglossomorph TAC1b that were basal to all teleost sequences. Preliminary synteny analysis on some available teleost genomes

(zebrafish, medaka) (data not shown) indicated that *tac1a* and *tac1b* genomic regions correspond to duplicated paralogs, supporting that the duplicated *tac1a* and *tac1b* genes are issued from the 3R and not from a local specific gene duplication. Synteny analysis of osteoglossomorph *tac3a* and *tac3b* genomic regions and comparison with the other teleosts would be required to confirm the classification of the paralogs in this group.

Duplicated *tac1a* and *tac1b* have been conserved throughout teleost radiation. Phylogeny analysis showed long branches for TAC1b pre-propeptide sequences in clupeocephala, suggesting larger sequence divergence of *tac1b*, as compared to *tac1a*. Furthermore, *tac1b* paralog would have been lost in some species, such as the stickleback and fugu, in the genomes of which we could identify only a single *tac1* gene. We classified their single gene as *tac1a*, according to the phylogeny analysis. This suggests independent and recurrent losses of *tac1b* paralog in some teleosts subgroups, gasterosteiforms and tetraodontiforms. Synteny analysis would allow us to further assess the loss of *tac1b* in these species.

Concerning the additional *tac1* genes identified in salmonids (up to four *tac1* genes), phylogeny analysis supports that they come from the additional whole genome duplication specific to the salmonid lineage (4R).

## **Two *tac4* genes and four TAC4 peptides in the eel**

We identified two *tac4* genes in the European eel, as well as in the American eel and Japanese eel. Each *tac4* gene encodes two predicted peptides. This organization of the TAC4 precursor coding for two peptides TAC and TAC-RP is a conserved feature as seen for human TAC4 pre-propeptide encompassing Endokinin (EK) (TAC4) and Hemokinin (HK) (TAC4-RP). The sequence of eel TAC4 peptides, TAC4a and b, TAC4-RPa and b are not common to any other studied TAC4 peptide and represent novel sequences.

The finding of two *tac4* genes in the eel is in agreement with the previous study by Zhou et al. (2012) who reported the presence of duplicated *tac4a* and *b* genes in zebrafish and medaka. We identified two *tac4* genes in most teleost species investigated, with the exception of osteoglossomorphs in which we retrieved only a single *tac4* gene (Figure 4 and Figure S2).

### ***Origin and fate of duplicated tac4a and b paralogs in teleosts***

In order to trace the origin and relationships of the teleost *tac4a* and *b* paralogs, we performed a phylogeny analysis of TAC4 pre-propeptide sequences. A single *tac4* gene was identified in chondrichthyan as well as in sarcopterygian species such as coelacanth or human. We also found a single *tac4* gene in the non-teleost actinopterygian, the spotted gar. In contrast, we found two *tac4* genes in the eel as well as in most other teleost species investigated. This suggests that duplicated *tac4a* and *b* paralogs may result from teleost 3R.

As described above for TAC1, phylogeny analysis of TAC4 pre-propeptide sequences showed that the spotted gar TAC4 branched at the base of all TAC4a and TAC4b teleost sequences, indicating a common actinopterygian ancestral *tac4* gene for teleost duplicated *tac4a* and *tac4b* genes. Furthermore, teleost sequences split into two clades, one clade encompassing all TAC4a pre-propeptide sequences and the other one all TAC4b pre-propeptide sequences. A previous synteny analysis of zebrafish *tac4a* and *b* genomic regions supports the 3R origin of teleost *tac4a* and *b* (Zhou et al., 2012).

Duplicated *tac4a* and *tac4b* have been conserved throughout teleost radiation, with the possible exception of osteoglossomorphs, in which we identified only a single *tac4* gene. Phylogeny analysis showed a basal position of this single *tac4* in the *tac4b* clade. Synteny analysis would allow us to further assess the loss of *tac4a* paralog in osteoglossomorphs. In contrast, as for *tac1*, we found up to four *tac4* genes in salmonids, likely resulting from 4R.



## ***Full complement of tachykinin genes and peptides in the eel***

In conclusion, we identified in a basal teleost, the European eel, two *tac1* genes encoding four TAC1 peptides, and two *tac4* genes encoding four TAC4 peptides. Our previous study also demonstrated the presence in the eel of two *tac3* genes, encoding four TAC3 peptides, and resulting from teleost 3R as shown by phylogeny and synteny analyses (Campo *et al.*, 2018). Altogether these results indicate that the eel has conserved all 3R-duplicated *tac* paralogs, leading to six *tac* genes and twelve peptides, as compared to three genes and five peptides in human. This provides novel peptide sequences for analyses of tachykinin structure activity relationships. As previously reported for TAC3 peptides (Campo *et al.*, 2018), future studies will aim at synthesizing eel TAC1 and TAC4 predicted peptides, and testing their biological activity in the eel.

As for tachykinin paralogs, we previously reported the remarkable conservation of duplicated paralogs issued from 1R/2R or 3R in the eel as compared to other teleost model species: for instance *hox* genes (Henkel *et al.*, 2012b); kisspeptins and receptors (Pasquier *et al.*, 2012), leptin and receptors (Morini *et al.*, 2015) and steroid receptors (Lafont *et al.*, 2016). The present results on tachykinins comfort the key position of the eel for tracing the evolutionary history of endocrine systems in teleosts. We are currently extending our investigation to the tachykinin receptors and our preliminary results indicate the presence in the eel of all 3R-duplicated paralogs of tachykinin receptors, leading to six receptors as compared to three in human. These studies open new research avenues for comparative evolutionary histories of tachykinin and tachykinin receptors across vertebrate and teleost radiation.

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**Table 1.** Sequences of European eel (*Anguilla anguilla*) predicted TAC1 and TAC 4 peptides and comparison with human peptides.

<b>Gene</b>	<b>Peptide name</b>	<b>Peptide sequence</b>
<i>Eel</i>		
tac1a	TAC1a	QKLNSFVSLM-NH2
	TAC1-RPa	PRPRQFFGLM-NH2
tac1b	TAC1b	HKLNSFVGLM-NH2
	TAC1-RPb	PRPHQFFGLM-NH2
tac4a	TAC4a	NKGEMFVGLM-NH2
	TAC4-RPa	SKSQQFHALM-NH2
tac4b	TAC4b	TKGEMFVGLM-NH2
	TAC4-RPb	SKSQQFYGLM-NH2
<i>Human</i>		
tac1	TAC1-RP (SP)	PKPQQFFGLM-NH2
	TAC1 (NKA)	HKTDSFVGLM-NH2
tac4	TAC4-RP (HK10)	TGKASQFFGLM-NH2
	TAC4 (EKC)	AYQLEHTFQGLL-NH2
	TAC4 (EKD)	VGAYQLEHTFQGLL-NH2

## **FIGURE LEGENDS**

**Figure 1:** Nucleotide and deduced amino-acid sequences of *tac1a* and *tac1b*. 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. Exons are indicated with a vertical line. The amino-acids of the signal peptide are underlined. The TAC1 peptides encoded by *tac1a* are in blue and by *tac1b* in red. Related peptides are in italic. The cleavage sites are marked by a square and amidation sites by a circle.

**Figure 2:** Nucleotide and deduced amino-acid sequences of *tac4a* and *tac4b*. 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. Exons are indicated with a vertical line. The amino acids of the signal peptide are underlined. The TAC4 peptides encoded by *tac4a* are in orange and by *tac4b* in green. Related peptides are in italic. The cleavage sites are marked by a square and amidation sites by a circle.

**Figure 3:** This phylogenetic tree was based on 90 deduced amino-acid sequences of *tac1* (see Fig S1) using the Maximum Likelihood method with 1,000 bootstrap replicates. The number shown at each branch node indicates the bootstrap value (%); only values and branching above 40% are indicated. The tree was rooted using the TAC1 sequence of the elephant shark *Callorhynchus milii*. The teleost TAC1a and TAC1b sequences are coloured in blue and red, respectively.

**Figure 4:** This phylogenetic tree was based on 90 deduced amino-acid sequences of *tac4* (see Fig S1) using the Maximum Likelihood method with 1,000 bootstrap replicates. The number shown at each branch node indicates the bootstrap value (%); only values and branching above 40% are indicated. The tree was rooted using the TAC4 sequence of the elephant shark *Callorhynchus milii*. The teleost TAC4a and TAC4b sequences are coloured in orange and green, respectively.

**Supplemental figure 1** : Alignment of 90 sequences of *tac1* gene collected and deduced from genome. Alignment was performed using CLUSTAL algorithm and manual reordering.

**Supplemental figure 2** : Alignment of 64 sequences of *tac4* gene collected and deduced from genome. Alignment was performed using CLUSTAL algorithm and manual reordering.

**FIGURES :**

**Figure 1 :**

TAC1a

```

1  ATGAAAATTACTACTGTCCATTTTGGTAGTTTTTCTTGCTCTGGCTGAAGTATTTTGTGAA  60
1  M K L L L S I L V V F L A L A E V F C E  20

61  GAAGTGGGCCAAAATGAAGATCTAAACCACTGGGCCAAACAATATTCAAATACAGGATGAG  120
21  E V G Q N E D L N H W A N N I Q I Q D E  40

121  TGGCTCGCTTCTGATCCACTAAACGAGATCCTGAAAAGAATCACAAGGAAACCTCGGCCT  180
41  W L A S D P L N E I L K R I T R K P R P  60

181  CGTCAGTTTTTTGGTCTAATGGGAAAGCGATCCTACGCAAATGCACAGATAACCCGCAAA  240
61  R Q F F G L M G K R S Y A N A Q I T R K  80

241  AGGCAAAAACCTCAACTCTTTTGTGAGCCTGATGGGGAAAAGAAGCCAGGAGGAGCCAATGAT  300
81  R Q K L N S F V S L M G K R S Q E E P D  100

301  ATGTATGAATGGAGAATACAGAACTAG  327
101  M Y E W R I Q N *  108

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TAC1b

```

1  ATGAAAATTACTCCTCTCTCTCGCGGTCGTTTTTCTTGTTCTGGCTGAAGTCTTTTGCGAA  60
1  M K L L L S L A V V F L V L A E V F C E  20

61  GAAATGAGTTCCGATGATGATCTCAATTACTGGACGAGAAGCAAAGAATTACAGGACGAC  120
21  E M S S D D D L N Y W T R S K E L Q D D  40

121  TGGTTTCCCGCGGGCCAGTTTCAGGAGATTCTGAGAAGGATGACGAGGAAGCCGCGGCCG  180
41  W F P A G Q F Q E I L R R M T R K P R P  60

181  CACCAGTTCTTTGGGTTAATGGGAAAGAGGTCTTCCCGCGCAAATGCACAGATGACCCGC  240
61  H Q F F G L M G K R S S A A N A Q M T R  80

241  AAAAGGCACAAGCTCAACTCCTTTGTGGGACTGATGGGAAAGAGGAGCCAAGAGGAGCCA  300
81  K R H K L N S F V G L M G K R S Q E E P  100

301  GATTCGTACGATTGGAGCAGAACGGACGAGGAATACCAACGGCGCTGA  348
101  D S Y D W S R T D E E Y Q R R *  115

```



Figure 2 :

TAC4a

1 <sup>Exon</sup> ATGGAAATTAAGTATTGGTTTTAATGGTTACTTTCTTTGCGCAAGTTTACTGCGTCCTG 60  
1 M E I K V L V L M V T F F A Q V Y C V L 20  
61 GGATCGTCGGCGAGCGATGATCGAGATTTCTGGCCGTCAGAGAACTGGCAG<sup>Exon</sup>GACGAGCCT 120  
21 G S S A S D D R D F W P S E N W Q D E P 40  
121 TTGGAGAACAGCCTGGCAACCCGCGTGGCAGATCTGATGAAGAGATCCAAATCGCAGCAG 180  
41 L E N S L A T R V A D L M **K R** **S K S Q Q** 60  
181 TTCCACGCGCTGATGGGCAGACGTTTCAG<sup>Exon</sup>GAGTTCCACAGCCCGTACGACTTGGTCCGAAAA 240  
61 **F H A L M** **G** **R R** S G V P Q P V R L G **R K** 80  
241 <sup>Exon</sup>AGAAACAAAGGAGAAATGTTTGTGGACTAATGGGAAGACGGTCATCAAGTGGAG<sup>Exon</sup>AGGTA 300  
81 **R** **N K G E M F V G L M** **G** **R R** S S S G E V 100  
301 CAAGAGGAGTTGGAGAAAACCCGTTTTACTAA 333  
101 Q E E L E K T P F Y \* 110

TAC4b

1 <sup>Exon</sup> ATGGATATCTGGAAACTCATTGTTTTAATAGTTTCATTTTTTTCGCTGATATACTGCACT 60  
1 M D I W K L I V L I V S F F A L I Y C T 20  
61 CAGGGATCATCTCCGAGCTATGAAAAGAAATACTGGTCGGCAGAGGGCTGGAAG<sup>Exon</sup>GACGAG 120  
21 Q G S S P S Y E K K Y W S A E G W K D E 40  
121 CCTTCGGAGAGCAGATTGGCTGGACGAGTGGTTGACCTCATAAAAAAGATCAAAATCTCAA 180  
41 P S E S R L A G R V V D L I **K R** **S K S Q** 60  
181 CAGTTCATGACTCATGGGAAGACGCTCAG<sup>Exon</sup>TAAAGCAGAAACCTGTCTTTAACCAAAAG 240  
61 **Q F Y G L M** **G** **R R** S V K Q K P V F N Q **K** 80  
241 <sup>Exon</sup>AGAACTAAAGGGGAGATGTTTGTGGCTCATGGGTAGGAGATCCTCAAGTGGAG<sup>Exon</sup>AGTTT 300  
81 **R** **T K G E M F V G L M** **G** **R R** S S S G E F 100  
301 CAAGAGGAATGGGACAAACATCAGTTTTACTGA 333  
101 Q E E W D K H Q F Y \* 110





# Supplemental Figure 1 :







**General discussion**

## ***V General Discussion***

### ***1 Multiple tac genes and peptides in the European eel***

Our studies have found six tachykinin genes in the European eel, two *tac1* genes, two *tac3* genes and two *tac4* genes. These genes are duplicated when compared with the three genes found in the spotted gar and in human. Our synteny studies revealed that the whole paralogon genomic region including the *tac3* gene has been duplicated in teleosts when compared to spotted gar. This duplication was most likely generated in the whole genome teleost duplication or 3R event reported previously (Meyer and Peer, 2005; Volff, 2005; Braasch and Postlethwait, 2012). Further synteny analysis developed on the *tac1* and *tac4* paralogs will assess the impact of the 3R on the duplication of these two genes.

Each of the tachykinin genes in the eel encoded two peptides, what makes a total of 12 tachykinin peptides. Four of those peptides belong to the *tac1* genes, four are encoded in the *tac3* genes and four are present in the *tac4* genes. However, not all human tachykinin genes encode two peptides. The human gene *tac3* encodes for a single tachykinin peptide, the neurokinin B (TAC3). The aminoacid sequence of this single TAC3 peptide is equal to all other studied sarcopterygian except the frog. It is also identical to the TAC3 peptide from the actinopterygian spotted gar and to the TAC3a peptide found in eel. Therefore, we can assume that the eel TAC3a peptide encoded in the *tac3a* gene is generally present in the sarcopterygian and ancestral actinopterygian species.

Interestingly, the other eel TAC3 peptides are different and unique in the *Anguilla* genus. The C-terminal ends conserve a full tachykinin formula, which indicates their capacity to activate the tachykinin receptors. On the other hand, the aminoacid sequences of the eel TAC3 peptides are divergent for the N-terminal end and have different lengths. While TAC3a and TAC3b are decapeptides, both TAC3RP peptides are made of 14 aminoacids. In addition, these related peptides present a tyrosine (Y) in the N-terminal end. This feature is conserved in most of the TAC3RP

peptides from the studied teleost species, the spotted gar, the coelacanth and in the elasmobranchs elephant shark (*Callorhynchus milii*), the small-spotted cat shark (*Scyliorhinus canicula*) and the little skate (*Leucoraja erinacea*). This conserved pattern in the N-terminal end indicates a possible role in the function of the tachykinins. In the human TAC1RP or substance-P, specific aminoacids indicating proteolytic sites are found in the N-terminal end (Regoli et al. 1994). These sites are different from those of the TAC1 peptide. Both peptides are encoded in the same gene and can be co-expressed. The differences in the aminoacid composition would induce different peptide degradation methods, which in turn produce different lifetime between the two peptides (for review: Severini *et al.*, 2002). This phenomenon has been described previously as a method for modulating the response of each tachykinin peptide when co-released with other tachykinins, as indicated by Maggi previously (2000). Therefore, the conserved tyrosine in the N-terminal of TAC3RP peptides and not found in the TAC3 peptides can also be a modulatory system for the tachykinin response. Post-transcriptional modifications in the N-terminal end must be also studied for a possible differential response between peptides.

None of the peptides encoded in the eel *tac1* and *tac4* genes are equal to the human peptides. TAC1RPb is a common peptide in the actinopterygian spotted gar and the basal teleosts, the eels and the arowanas. Those peptides are also in common with the TAC1RPa that is encoded in the *tac1a* gene of northern pike and the studied salmonids species. The eel TAC1b is also in common with the TAC1a from rainbow smelt, *Osmerus mordax*. These results indicate a convergence between genes *tac1b* from eel and *tac1a* from *osmeriformes*, *esociformes* and *salmonids*. This convergence needs to be verified by further synteny analysis of the *tac1* genomic region.

While the peptides TAC4RPa and TAC4b are unique to the eel species, TAC4a and TAC4RPb are shared with other species. TAC4RPb is common to the eels and the basal teleosts bony tongue elephantfish (*Paramormyrops kingsleyae*) and pirarucu (*Arapaima gigas*). Therefore, eel TAC1RPb, and TAC4RPb have a conserved sequence.



Interestingly, TAC4a is conserved between most of the studied teleost species including the eel (Article 2, table1). Another teleost tachykinin, TAC3a peptide found in Atlantic herring with sequence EMHDI FVGLM-NH<sub>2</sub> is also conserved by many of the *clupeocephala* species used in our analysis, but not in the eels or the arowanas (Article 1, Supplemental Figure 1). These findings indicate that the tachykinin peptides in teleosts have possibly developed a particular functioning for TAC3a and TAC4a peptides that has been conserved strictly among the wide teleost radiation. In contrast, for some species such as northern pike and zebrafish, the TAC3b peptide shows a substitution of the final methionine (M) by a similar aminoacid, the leucine (L). Other teleost species TAC3b have a non-tachykinin C-terminal end (LADLL-NH<sub>2</sub> in the perch *Perca fluviatilis*; LAALL-NH<sub>2</sub> in sea bass *Dicentrarchus labrax*; LAELL-NH<sub>2</sub> in the barramundi *Lates calcarifer*). The differences in the sequence maybe associated with the different pattern of receptor recognition and activation (Jakob Biran *et al.*, 2012).

In the eel species, the peptides TAC1a and TAC4RPa present different tachykinin motives. TAC1a has a single substitution of the glutamine (G) by serine (S), being the C-terminal formula FVSLM-NH<sub>2</sub>. TAC4RPa presents a substitution of G by alanine (A) in the tachykinin motive FHALM-NK<sub>2</sub>. The second position of this C-terminal end has an aromatic aminoacid, the histidine (H), as reported previously for other tachykinin peptides (Severini *et al.*, 2002). We can assume that the peptides TAC1a and TAC4RPa are divergent tachykinin-like peptides, exclusive to the *Anguilla* genus. These sequences must be verified by cloning.

The mid regions of the TAC1 and the TAC4 peptides have similar aminoacids marked with the presence of one or two glutamines (Q), a non-polar aminoacid (Article 2, Table 1). In contrast, TAC3 peptides are characteristic for including one or two aspartic acids (D) in this same mid regions (Article 1, Table 1). According to our modelling results, this aminoacid is also involved in the alpha-helix structure of the eel peptides and is placed also in a highly hydrophobic area of the peptide. Differently from glutamine, the aspartic acid has a negative charge that may affect the charge of the

whole TAC3 peptide. This charge may be a particular indicator for receptor recognition and affinity, which may explain the preference of the mammalian TAC3 receptor for TAC3 peptides when compared to the other tachykinin peptides (Buck and Burcher, 1986).

The new tachykinin peptides found in the eel open the possibility for different combinations of peptide-receptor. Differential selectivity of all tachykinin receptors has been reported for the human TACRP1 (substance P), TAC1 (Neurokinin A) and TAC3 (Neurokinin B) and TAC4RP (Hemokinin) peptides (Buck and Burcher, 1986; Morteau *et al.*, 2001; for review: Regoli *et al.*, 1987; Quirion and Dam, 1988; Maggi, 1995; Pennefather *et al.*, 2004; Borbély and Helyes, 2017). As suggested previously for kisspeptin and its receptors (Pasquier *et al.*, 2014), the conservation of the receptor activation site in the C-terminal end of the peptide, may facilitate certain flexibility of peptide/receptor association in the tachykinin systems along the vertebrate radiation. Therefore, the peptides found in the eel and in the studied teleost species can enlarge the already wide pharmacological possibilities of the tachykinins.

## ***2 Evolutionary history of the tachykinins***

We found that the conservation of all the duplicated genes in the eel applies also to the Japanese and the American eel. Previous analysis of the hox cluster demonstrated already a wide conservation of the genes in the European eel genome (Henkel *et al.*, 2012a), that was confirmed also for the Japanese eel (Henkel *et al.*, 2012b). Other genes have been conserved in duplicate in the eel after the 3R: dopamine D2 receptors (Pasqualini *et al.*, 2009), GnRH-receptor type I (Peñaranda *et al.*, 2013),  $\beta$ -subunit of thyroid-stimulating-hormone and its receptor (Maugars *et al.*, 2014), leptin (Morini *et al.*, 2015), progesterin receptor (Morini *et al.*, 2017), intracellular nuclear receptor-2 and G-coupled membrane receptors for estrogen (Lafont *et al.*, 2016; Morini *et al.*, 2017). Therefore, there are evidences that the eel is a particular teleost with a considerable retention of the teleost duplicated genes and in particular the tachykinins.



However, our results indicate that many teleosts have conserved the duplicated tachykinin peptides after the 3R: northern pike, zebrafish and tilapia, among others. During our research we discovered a *tac1b* gene in zebrafish and a *tac3b* gene in tilapia that were not reported in previous studies (zebrafish: Zhou *et al.*, 2012; tilapia: Biran *et al.*, 2014). Hence, the multiple-gene loss for genome reshaping after the 3R (Inoue *et al.*, 2015) didn't affect the tachykinin genes in teleosts.

According to the phylogeny studies, the pre-pro-tachykinin peptide that is encoded in the *tac3b* gene is divergent for the clade of neoteleosts, where tilapia belongs. Species within this group have highly divergent TAC3b peptides: no signs of the tachykinin motive, the cleavage sequence and/or the amidation site (fugu, stickleback, the mangrove killifish *Kryptolebias marmoratus*, the zebra-mbuna *Maylandia zebra* and the pundamilia *P. nyererei*). These species would have lost the possibility to cleave the TAC3b peptide or to amidate it in a sort of non-functionalization event possibly due to lack of selective constraint as reported for teleost duplicated genes (Glasauer and Neuhaus, 2014) . However, the TAC3RPb includes a complete tachykinin peptide with cleavage sequence and amidation signal, which may be translated and could prevent the disappearance of the *tac3b* gene.

Other species show a different C-terminal with substitution of the final methionine by a leucine (zebrafish, goldfish *Carassius auratus*, northern pike and cavefish *Astyanax mexicanus* among others). The phylogenetic tree indicates that this paralog *tac3b* is more divergent than the other paralogon. Therefore, *tac3b* follows a more accelerated evolutionary rate than the paralog *tac3a*, in agreement with previous studies on paralogs after the teleost whole genome duplication (Brunet *et al.*, 2006).

Similarly, *tac1* gene is paralog to *tac4* from the second round of genome duplication events (2R) that underwent the vertebrate lineage. The human TAC4 peptide has a substitution of methionine by leucine in the tachykinin C-terminal end (Naono *et al.*, 2007). In this case, the peptide TAC4 developed a new function by activating the same receptor than the TAC1RP peptide. While TAC4RP

induces hyperalgesia, TAC4 counteracts its activity in an analgesic action (Yang and Dong, 2010; for review: Borbély and Helyes, 2017). The promiscuity of the tachykinin receptor and its different responses could ease the neofunctionalization of the tachykinin peptides. Further studies on the eel tachykinin receptors may be of interest for understanding the function of each tachykinin peptide in the eel.

The tachykinin genes have been pretty conserved in sarcopterygians and especially in mammals. Losses are mostly found in the *tac4* gene, for the frog, snakes and birds, but not in turtles. In addition, the *tac3* gene is preserved in the chicken, the great tit *Parus major*, the quail *Coturnix japonica* and the blue-crowned manakin *Lepidothrix coronata*. However, it is lost on other species as the duck *Annas platyrhynchos*. This indicates that the tachykinin genes in sarcopterygians might undergo independent losses. In addition, the *tac1* gene is the most widely present in the sarcopterygian lineage.

### ***3 Reproductive role of the TAC3 in the eel***

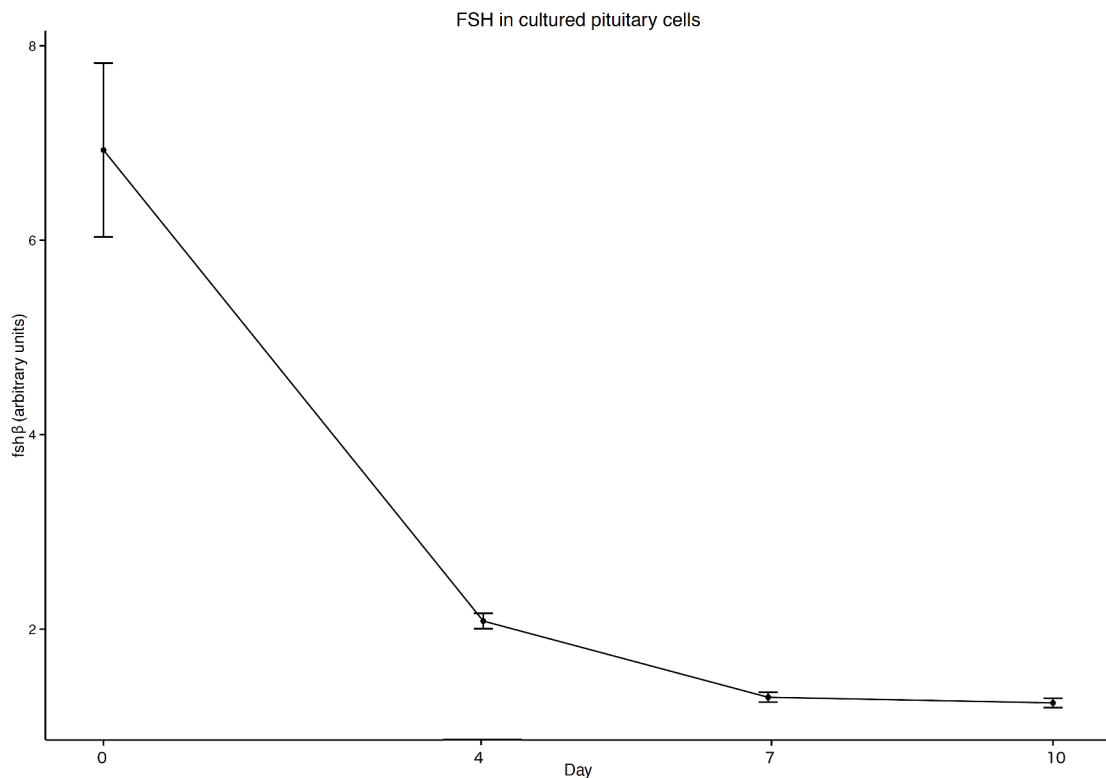
#### ***3.1 Effect on pituitary gonadotropins***

Our experiments *in vitro* resulted in a dual inhibition of the mRNA expression of gonadotropin  $\beta$ -subunit, *lh $\beta$* , and the receptor II of GnRH, *gnrh-rII*. The dual regulation of *lh $\beta$*  and *gnrh-rII* in the eel pituitary has been reported also for kisspeptin (Pasquier *et al.*, 2018). These results reveal a direct regulation by the TAC3 peptides on pituitary gonadotropins of the pre-pubertal silver eel. The peptide product of the genes *lh $\beta$*  and *gnrh-rII* work together during the sexual maturation regulating the inhibition process in the silver eel in two different manners: by decreasing the gonadotropin level and by reducing the sensitivity to GnRH. Further *in situ* analysis of the *lh $\beta$*  and *gnrh-rII* genes expression will demonstrate co-expression of these genes in the same cells or in different cell-types.

The *in vitro* effect of TAC3 peptides in the pituitary gonadotropins has been described for other teleosts species (tilapia: Biran *et al.*, 2014; Jin *et al.*, 2016; striped bass: Zmora *et al.*, 2017; orange

spotted grouper: Chen *et al.*, 2018). Studies in grass carp revealed the effect of TAC3 peptides on somatolactin- $\alpha$  expression mediated by TACR3, prolactin expression mediated by TACR2 and also the translation and the release of the prolactin (Hu *et al.*, 2014a). The peptides encoded in the *tac1* gene induced the release of somatolactin- $\alpha$  and prolactin, and interestingly, also the release of LH. The TAC1RP peptide induced the release of LH after 3 hours of activity and the attenuation of *lh $\beta$*  expression after 24h exposition to this peptide (Hu *et al.*, 2017). This activity on LH was mediated by the receptor TACR1. Our preliminary tests on the pre-pubertal silver eel pituitary cells determined that the TAC3 peptides do not have any effect on prolactin in cultured pituitary cells. Further research with the discovered eel peptides encoded in the *tac1* and *tac4* genes will assess other possible direct effect of the tachykinin peptides on pituitary expression.

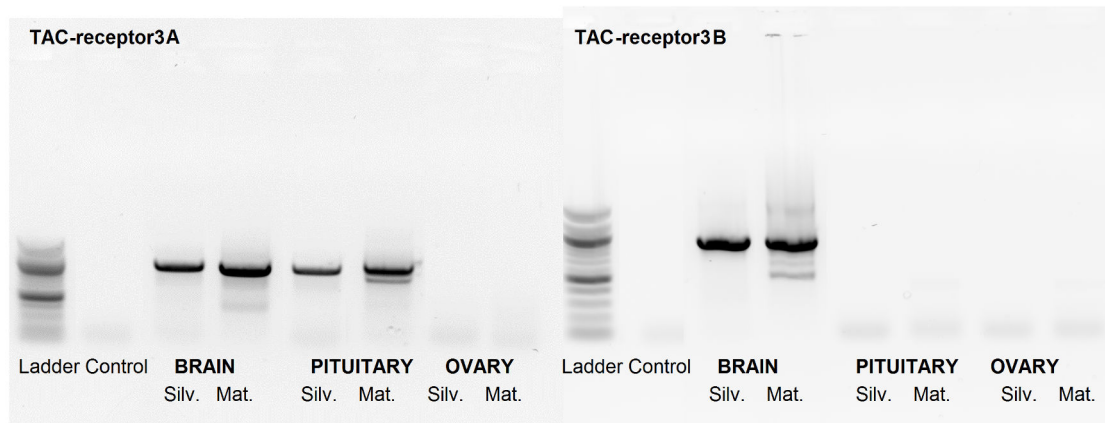
The TAC3 peptides inhibited the expression of *lh $\beta$*  but didn't affect the expression of *fsh $\beta$* . The expression of *fsh $\beta$*  in the pituitary cell culture revealed a pattern of decrease in all the experimental groups and the control after 4 days in culture (Figure 10). This pattern can indicate an *in vivo* stimulation of *fsh $\beta$*  in the pre-pubertal silver eel. Because the *fsh $\beta$*  expression maybe was very low during the cell culture, the possible inhibitory effect of the TAC3 peptides couldn't be assessed. Next experiments may be needed to look for the response of *fsh $\beta$*  in presence of a stimulator such as activin to counteract the natural inhibitory phenomena.



**Figure 10:** *Fshβ* expression in the cultured pituitary cells. Control group.

In order to assess the possible direct effect on gonadotropin cells, studies on the expression and activation of tachykinin receptors are necessary. *In situ* hybridization of the TACR3 indicated that it is co-expressed with *lhβ* in the pituitary of tilapia (Biran *et al.*, 2014a) thus indicating the direct effect on gonadotropin. In addition, *in situ* hybridisation in carp pituitary cells revealed that tachykinin receptors may be specialized in the direct regulation of secretion for gonadotropes (TACR1) lactotropes (TACR2) and somatotropes (TACR3) (Hu *et al.*, 2017).

Our preliminary analysis by PCR of both eel *tacr3* receptor genes indicate that they are both expressed in the brain, but only one paralog, the *tacr3a*, is likely expressed in the pituitary (Figure 11). We can then raise the hypothesis that *tacr3a* paralogon and not *tacr3b* is implicated in the inhibitory effect of TAC3/TAC3RP peptides in *lh* and *gnrh-rii* on the pituitary. Further analysis by *in situ* hybridisation in the eel will be of interest for revealing the possible co-expression of the *tacr3* genes with the *lhβ* subunit.



**Figure 11:** PCR analysis of the *tacr3a* and *tacr3b* in the brain, pituitary and ovary of two European eel individuals, one of them silver (Silv.) and the other artificially matured (Mat.).

### 3.2 Effect on the brain

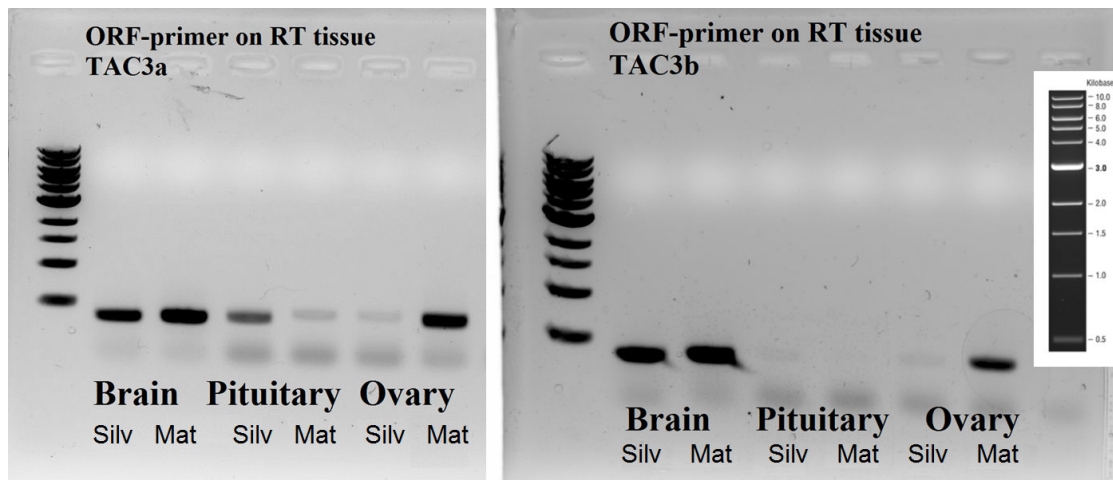
The brain distribution of the eel *tacr3* genes indicates that there is a wide expression of the two paralogs in the diencephal (Article 1, Figure 4). Kisspeptin peptides, in particular Kiss2 have also shown to be widely expressed in this area (Pasquier *et al.*, 2018), where the three GnRH-receptors of the eel are also expressed (Peñaranda *et al.*, 2013). In mammals, the diencephalon includes the arcuate nucleus (for review: Hu *et al.*, 2014b) where the KNDy neurons are found (Mittelman-Smith *et al.*, 2012). The possible action of tachykinin and kisspeptin peptides on GnRH in teleosts would be hence analogue to the one of the mammal KNDy neurons. Kiss and TAC3 neurons are expressed in distinct neurons in the striped bass but TAC3 peptides downregulated the expression of Kiss2 thus reducing the content of GnRH1 in the pituitary (Zmora *et al.*, 2017).

The preliminary finding of the expression of *tacr3*, both paralogs, in the brain (Figure 11) opens the possibility for a local action on expression or release of GnRH, involving or not Kiss2, in a direct or indirect manner. Further analysis of tachykinins and their receptors in the diencephal area will be of interest for understanding the tachykinergic role in the diencephal and the possible regulation of GnRH release to the pituitary.



### 3.3 Effect on the gonads

In silver pre-pubertal eel, qPCR analysis on *tac3* gene distribution in the ovary shows the weak presence of the expression of *tac3a* gene and no presence of the *tac3b*. Nevertheless, the PCR analysis for a preliminary tissue distribution on artificially matured female eel identified both paralogs in the ovary (Figure 12).



**Figure 12:** PCR preliminary tissue distribution of the *tac3* genes in one mature (Mat.) and one silver stage (Silv.) European eel individuals.

The role of tachykinin peptides in the ovary have been determined in the ascidian *C. intestinalis* (Aoyama *et al.*, 2008). The ascidian has a single tachykinin gene encoding two peptides and a single tachykinin receptor. The TACR is present in the ovary and the TAC peptides are necessary for progression from vitellogenic to post-vitellogenic stage by increasing the expression of proteases and increasing also their enzymatic activities. Studies in the ovary function of *tac3* and the *tacr3* genes revealed a role of the human TAC3 peptide in the acceleration of follicle development, upregulation of the aromatase genes and an increase of the estradiol production *in vivo* and *in vitro* on zebrafish (Qi *et al.*, 2016). Human TAC3 peptide applied *in vitro* to human granulosa cell line upregulated also the expression of aromatase in the same studies. In mouse, the steroids produced in the ovaries regulate the presence of the tachykinin peptides in the uterus (Pinto *et al.*, 2009); the role in the uterus may be related with contractility (Patak *et al.*, 2003). Recent studies determined that the

whole tachykinin system, including *tac1* and *tac4* peptides and all three receptors is expressed in the human granulosa cells and in the cumulus cells for regulation of the ovary function. In addition, the tachykinin expression and effect is modulated by kisspeptin (García-Ortega *et al.*, 2016).

Our preliminary analysis in the eel suggests that both paralogs *tac3a* and *tac3b* are more expressed in mature ovaries than in ovaries of the pre-pubertal stage. Therefore, eel tachykinins may have a potential role in gonads maturation. Since our preliminary PCR study did not detect any *tacr3* in the ovary (figure 11), the function of the TAC3 peptides may be exerted *via* another receptor. The characterization of the tachykinin system in the ovary of the eel will open a new aspect of the eel sexual maturation process.

### **Perspectives**

## ***VI Perspectives***

The tachykinin peptides characterized in the European eel during this thesis is the beginning of a long experimental path till full understanding of the tachykinin system in the eel. The next projects will allow a wider comprehension of this system in the eel, the vertebrates radiation and the sexual maturation process.

### ***1 Tac1 and 4 in the eel***

For completing the characterization of the new tachykinin peptides discovered in the eel it will be necessary to investigate the localization of expression by qPCR (tissue distribution).

The synthesis of the four predicted TAC1 peptides and the four predicted TAC4 peptides of the eel, will allow to test their biological effects on eel pituitary cell culture to investigate the possible tachykinin regulations on gonadotropins or other pituitary hormones and receptors.

### ***2 Tac receptors in the eel***

The *in silico* characterization of the tachykinin receptors in spotted gar and in the eel was initiated during this thesis. As in human, three tachykinin receptors were identified in the gar. Consistently, six tachykinin receptors were found in the genome of the three eel species, likely generated in the 3R. Further synteny analysis must be performed to assess the impact of the whole genome duplication event in the whole tachykinin system. In addition, sequences must be verified by cloning.

Phylogeny studies must be performed for comparative research of the tachykinin system in vertebrates and in teleost radiation.

Later, preparation of qPCR assay for tissue distribution of those 6 receptors will characterize the potential target tissues of the tachykinin system in the eel.

### ***3 Potential receptor/ligand couples in the eel: recombinant receptors and test of peptides***

In order to determine receptor preferences for ligand, experiences the six recombinant eel tachykinin receptors will be expressed in cell lines (CHO) cells must be carried.

### ***4 Comparative evolutionary history of tac and tac receptors***

A comparative evolutionary history of the tachykinin and receptors will reveal the conservation and loss of tachykinins and receptors in vertebrate key species.

This analysis can also reveal the possible couples of peptide and receptor that are bound in a co-evolution constraint.

To test the ability of the twelve eel TAC peptides to bind to the receptors and activate second messenger pathways.

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# Characterization of tachykinin system and role in reproduction in the European eel

## Résumé

L'objectif de cette thèse est d'étudier le rôle de neuropeptides cérébraux, telle que la Neurokinin B codée par le gène *tac3*, dans le contrôle de la reproduction d'une espèce en danger, l'anguille Européenne, *Anguilla anguilla*. La maturation sexuelle de l'anguille est bloquée à un stade prépubertaire avant la migration océanique. Etant donnée sa position phylogénétique basale parmi les téléostéens, l'anguille est un modèle pertinent pour étudier l'évolution moléculaire et fonctionnelle de neuropeptides d'intérêt. Deux gènes paralogues tachykinine 3 (*tac3*) ont été identifiés dans le génome de l'anguille, chacun codant pour deux peptides. Ces gènes paralogues résultent de la duplication complète du génome spécifique aux téléostéens, comme le montrent les analyses phylogénétiques et synténiques. Les analyses de qPCR montrent que les deux gènes sont exprimés dans le cerveau. Les quatre peptides d'anguille ont été synthétisés et testés sur des cultures primaires de cellules hypophysaires d'anguille. Les quatre peptides inhibent l'expression de l'hormone lutéinisante et d'un récepteur à la gonadolibérine, révélant un double rôle inhibiteur dans le contrôle de la reproduction.

Mots clés : Tachykinine, *Anguilla*, téléostéens, évolution, reproduction

## Abstract

The aim of this PhD is to investigate the role of brain neuropeptides, such as neurokinin B, encoded by *tac3* gene, in the control of reproduction of an endangered species, the European eel, *Anguilla anguilla*. The sexual maturation of the eel is blocked at a prepubertal stage before the oceanic migration. Due to its basal phylogenetic position among teleosts, the eel is also a relevant model for studying molecular and functional evolution of key neuropeptides. Two tachykinin 3 (*tac3*) paralogous genes were identified in the eel genome, each encoding two peptides. These paralogs result from the teleost-specific whole genome duplication, as shown by phylogeny and synteny analyses. Both genes are expressed in the brain as shown by qPCR. The four eel peptides were synthesized and tested on primary cultures of eel pituitary cells. The four peptides inhibited the expression of luteinizing hormone and gonadotropin-releasing hormone receptor, revealing a dual inhibitory role in the control of reproduction.

Key-words: Tachykinin, *Anguilla*, teleost, evolution, reproduction