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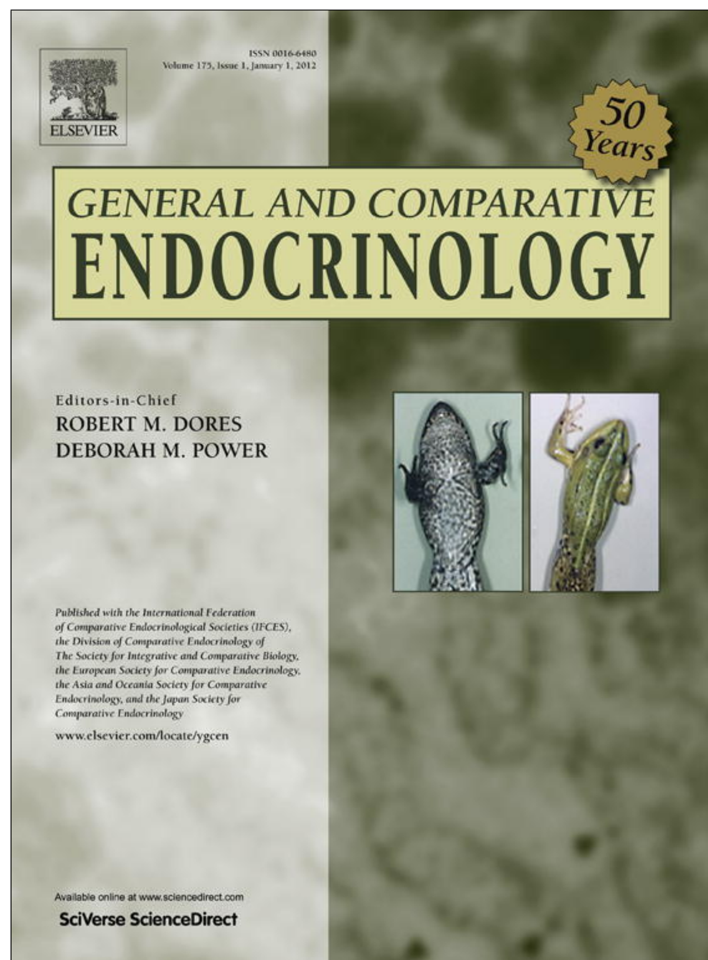

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Pituitary gonadotropins FSH and LH are oppositely regulated by the activin/follistatin system in a basal teleost, the eel

Salima Aroua [a,1], Gersende Maugars [a,1], Shan-Ru Jeng [b], Ching-Fong Chang [c], Finn-Arne Weltzien [d], Karine Rousseau [a], Sylvie Dufour [a,*]

[a] Laboratory of Biology of Aquatic Organisms and Ecosystems (BOREA), UMR CNRS 7208-IRD 207-UPMC, Muséum National d'Histoire Naturelle, 7 rue Cuvier, CP 32, 75231 Paris Cedex 05, France
[b] Department of Aquaculture, National Kaohsiung Marine University, Kaohsiung 814, Taiwan
[c] Department of Aquaculture, Center of Marine Biosciences and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan
[d] Department of Basic Sciences and Aquatic Medicine, Norwegian School of Veterinary Science, P.O. Box 8146 Dep, 0033 Oslo, Norway

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ABSTRACT

European eels are blocked at a prepubertal silver stage due to a deficient production of pituitary gonadotropins. We investigated the potential role of activin/follistatin system in the control of eel gonadotropins. Through the development of qPCR assays for European eel activin β_B and follistatin, we first analyzed the tissue distribution of the expression of these two genes. Both activin β_B and follistatin are expressed in the brain, pituitary and gonads. In addition, a striking expression of both transcripts was also found in the retina and in adipose tissue. The effects of recombinant human activins and follistatin on eel gonadotropin gene expression were studied using primary cultures of eel pituitary cells. Activins A and B strongly stimulated FSHβ subunit expression in a time- and dose-dependent manner. In contrast, activin reduced LHβ expression, an inhibitory effect which was highlighted in the presence of testosterone, a known activator of eel LHβ expression. No effect of activin was observed on other pituitary hormones. Follistatin antagonized both the stimulatory and inhibitory effects of activin on FSHβ and LHβ expression, respectively. Activin is the first major stimulator of FSH expression evidenced in the eel. These results in a basal teleost further support the ancient origin and strong conservation of the activin/follistatin system in the control of FSH in vertebrates. In contrast, the opposite regulation of FSH and LH may have emerged in the teleost lineage.

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1. Introduction

The pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are glycoprotein hormones comprised of two subunits, α (GPα) and β. While GPα is common for both hormones, β is hormone specific and confers the biological activity to each hormone. FSH and LH are important components of the brain–pituitary–gonad axis, the neuroendocrine axis involved in the control of reproductive function in vertebrates. FSH and LH play their actions on the gonads by inducing gametogenesis and steroidogenesis. In turn, the sexual steroids regulate brain and pituitary factors through differential feedback mechanisms [2,30]. In vertebrates, gonadotropin production and release are under the stimulatory control of brain gonadotropin-releasing hormone (GnRH). In addition, in some teleosts, a brain inhibitory control is exerted by dopamine (DA) [21,22].

Besides these control elements, gonadal peptides play an important role in the regulation of gonadotropins in mammals [82]. Activin was first purified from porcine ovarian fluid for its ability to stimulate FSH synthesis and release from cultured pituitary cells [13,45,77]. Activins are members of the transforming growth factor-β (TGF-β) superfamily, and are disulfide-linked homo- or hetero-dimers of two β subunits: activin A ($\beta_A\beta_A$), activin B ($\beta_B\beta_B$) and activin AB ($\beta_A\beta_B$) [76,82]. Additional activin β_C, β_D and β_E subunit genes have been recently identified in some vertebrates [42]. While activin β_A and β_B subunit mRNA present a broad expression pattern [9,51], β_C and β_E mRNAs are predominantly expressed in the liver [42]. During the purification of activin, an activin-binding protein named follistatin was also isolated and characterized [64,75]. Follistatin was found to neutralize activin bioactivity [58]. The effects of these two gonadal peptides on FSH in mammals were found to be specific, as no changes were observed on LH [33]. Similar to activin genes, follistatin is detected not only in gonads, but also in a wide range of tissues including the pituitary, where its expression has been localized in gonadotrope cells [40,52]. These observations suggest that in mammals, the actions of activin and follistatin could be exerted locally as autocrine/paracrine effects on gonadotropins.

* Corresponding author. Address: MNHN – UMR BOREA, 7 rue Cuvier, CP 32, 75231 Paris Cedex 05, France. Fax: +33 (0) 1 40 79 36 18.
E-mail address: dufour@mnhn.fr (S. Dufour).
[1] These authors contributed equally to this work.

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In teleosts, the first data on the existence of the activin-like system and its potential role on gonadotropins were reported in goldfish by Ge and collaborators. They showed that treatments of goldfish pituitary cells with porcine activin A induced an increase in gonadotropin release [25]. This group later demonstrated that recombinant goldfish activin B could differentially control gonadotropins by increasing FSHβ and reducing LHβ gene expression in goldfish [80] and zebrafish [44] pituitary cells. However, preliminary studies in two other teleost species did not report such an opposite action of activin on FSH and LH. In tilapia cultured pituitary cells, recombinant human activin A induced an increase in all gonadotropin subunit mRNA [81]. In coho salmon pituitary cells, recombinant human activin A induced an increase in mRNA levels of both GPα and FSHβ, with no effect on LHβ [16]. Concerning follistatin, first works performed on cultured pituitary cells of goldfish have shown that recombinant porcine follistatin was able to counteract the effects of activin on FSH and LH expressions [83].

Eels have a complex migratory life cycle with reproduction in the ocean and long juvenile growth phase in continental waters. Furthermore, they remain blocked at a prepubertal stage (silver stage) as long as the reproductive oceanic migration is prevented. This blockade is due to deficient production of pituitary gonadotropins [20,22]. A main aim of endocrine investigations in the eel is to find factors able to stimulate the expression and release of gonadotropins. Concerning LH, various factors such as testosterone, cortisol and insulin-like growth factor-1 (IGF-1) were shown to induce LH synthesis and release *in vitro* in the European eel (*Anguilla anguilla*) [36–38]. In contrast, up to now, no strong stimulatory factor has been found for FSH. The comprehension of mechanisms controlling reproduction in the eel is of particular interest considering the dramatic decline of wild populations [69] and the present impossibility to reproduce European eel in captivity. Moreover, as the eel is a representative species of a phylogenetically ancient group of teleosts (elopomorphes) [18,39], understanding the gonadotropin regulation in this species will provide information on ancestral regulatory mechanisms.

The aim of the present study was to investigate the potential role of the activin/follistatin system in the control of eel gonadotropin expression. Activin β_B was cloned in 1999 from Japanese eel (*Anguilla japonica*) testis [53]. We partially cloned activin β_B and follistatin from European eel brain and developed quantitative real-time PCR (qPCR) assays to study their tissue distributions. Using primary culture of eel pituitary cells, we analyzed the direct effects of recombinant human activin A and B, and follistatin in the control of FSHβ and LHβ subunit gene expression.

2. Materials and methods

2.1. Animals

Female European eels were netted during their downstream migration (prepubertal silver stage) by professional fishermen in the Loire river. Animals were transferred to the laboratory (MNHN, Paris, France) and kept in running aerated freshwater tanks under natural photoperiod and temperature (15 °C). Because eels are naturally fasting at the silver stage, they were not fed. Animal manipulations were performed according to French regulations and the European convention on animal experimentation for scientific research.

2.2. Hormones

Recombinant human activin A and B and follistatin were purchased from R&D system (Lille, France). Testosterone (T) was purchased from Sigma (Lyon, France).

2.3. Sample collection for tissue distribution

Tissues were collected from eight female silver eels to investigate the distribution of activin β_B subunit and follistatin expression. Eels were sacrificed by decapitation. The following organs were quickly removed, stored in RNAlater (Ambion Inc., Austin, TX, USA) and kept frozen at −20 °C until extraction: brain, pituitary, ovary, retina, muscle, intestine, liver, gill and adipose tissue. Brain was dissected into five parts according to Sébert et al. [66]: olfactory bulbs, telencephalon, di-/mes-encephalon, cerebellum and medulla oblongata.

2.4. Tissue RNA extraction and cDNA synthesis

Tissue samples were homogenized by sonication in Trizol and total RNA were extracted according to the manufacturer's instructions (Invitrogen SARL, Cergy Pontoise, France). Following extraction, samples were treated with DNase I (Roche, Meylan, France), and the first strand of cDNA was synthesized from 400 ng of total RNA using Superscript III reverse transcriptase (Invitrogen) and random hexamer primers. The reaction was performed according to the following thermal conditions with an initial step at 25 °C for 10 min followed by incubation at 50 °C for 60 min and 70 °C for 15 min.

2.5. Primary culture of eel pituitary cells

2.5.1. Cell dispersion and primary culture

Dispersion of pituitary cells was performed using an enzymatic and mechanical method as described by Montero et al. [55]. Briefly, 20–25 pituitaries were collected for each cell preparation, washed in calcium and magnesium free saline phosphate buffer (Ca/Mg free PBS) (Gibco, Invitrogen) and cut into 1 mm slices. Slices were incubated for 1 h at 25 °C in a solution of porcine type II trypsin (Sigma). The reaction was stopped by replacement of the trypsin solution by a solution of DNase II (Sigma) and soy bean trypsin inhibitor (Sigma) for 10 min. Pituitary slices were then washed in Ca/Mg free PBS and mechanically dispersed by repeated passages through a plastic transfer pipette (Falcon, Dutscher, Brumath, France). After estimating the number of viable cells by trypan blue exclusion (Sigma), cells (50,000 cells/well) were plated in 96-well plates precoated with poly-L-lysine (Sigma). Cultures and treatments were performed in serum-free culture medium (Medium 199 with Earle's salt, sodium bicarbonate, 100 U/ml penicillin, 100 μl/ml streptomycin, 250 ng/ml fungizone (Gibco)) at 18 °C under 3% CO_2 and saturated humidity.

2.5.2. In vitro treatments

Stock solutions of recombinant human activin B (5×10^{-5} M), recombinant human activin A (5×10^{-5} M) and recombinant human follistatin (5×10^{-6} M) were prepared in sterile PBS (Gibco), and testosterone stock solution (10^{-3} M) was prepared in ethanol. Hormonal treatments started 24 h after plating to allow cell attachment. Culture medium was changed and hormones were added to wells on Day 0, and renewed on Day 3 and Day 6. Stock solutions were diluted in culture medium just before their addition into the culture wells. Control wells were treated with similar solvent dilutions of ethanol or PBS. Cultures were stopped either on Day 3 or Day 9. Five replicate wells were used for each treatment and treatments were performed at least in three independent experiments on different cell preparations from independent batches of fish. Each figure displays the results of a representative experiment.

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2.5.3. Cell RNA extraction and cDNA synthesis

RNA extraction and cDNA synthesis from cultured cells were performed according to the method previously described by Aroua et al. [4]. Total RNA was directly extracted from each culture well using the cell-to-cDNA I kit (Ambion Inc.). After washing with sterile PBS, cells were lysed with the Cell Lysis II buffer (80 μl/well), and the lysates were submitted to a DNase I treatment (Ambion Inc.). Reverse transcription was performed as described above (Section 2.4) using 5 μl cell lysate.

2.6. Quantitative real-time PCR (qPCR) assays

2.6.1. Partial amplification and cloning of European eel activin β_B and follistatin cDNA

As a basis for qPCR primer design, European eel activin β_B and follistatin were partially cloned and sequenced. Total RNA was isolated from eel brains and DNase-treated as described in Section 2.4. First-strand cDNA was synthesized from 1 μg of total RNA using oligo(dT)$_{20}$ primers (Invitrogen) and Superscript III reverse transcriptase (Invitrogen). PCR primers for each gene were designed from the corresponding Japanese eel sequences (Accession No. activin β_B AB025356 and follistatin JF681369) using Primer3 [65] and vector NTI (Invitrogen) and were manufactured by Eurofins MWG Operon (Germany). The primers were for activin β_B (Fw 5′-atgaaaaggtattgtgtcaaagtgg-3′, Rv 5′-cctcgttggagatgaggaaa-3′) and for follistatin (Fw 5′-gctggctgcagcaggggaataa-3′, Rv 5′-gacatatctcgccacacac-3′). PCR fragments for both genes were amplified using AccuPrime *Taq* DNA polymerase (Invitrogen) in a reaction mixture of 25 μl containing 500 nM of each primer and 1 μl of cDNA template. For both DNA fragments the PCR amplification was run as follows: denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 50 °C for 15 s, and 68 °C for 2 min, and finally an extension step at 68 °C for 5 min. PCR products were visualized on agarose gel and bands of expected size were purified using Qiaquick gel extraction kit (Qiagen) and ligated into the pGEM-T easy vector (Promega, WI, USA). Cloning was performed in competent *Escherichia coli* JM109 cells (Promega). Positive colonies were isolated and plasmids were extracted by a plasmid mini kit (Qiagen). Plasmids with insert were sequenced using an ABI 3730 DNA analyzer (Applied Biosystems, University of Oslo sequencing platform, Oslo, Norway).

Amplification of European eel activin β_B resulted in a DNA fragment of 438 bp which includes parts of exons 1 and 2 (Accession No. GU269543). Amplification of European eel follistatin resulted in a DNA fragment of 729 bp containing exons 2–5 (Accession No. FR839633). These sequences showed 98% and 92% nucleotide identities with Japanese eel activin β_B and follistatin, respectively.

2.6.2. qPCR primers (Table 1)

Specific primers for European eel activin β_B and follistatin were designed based on the partially sequenced fragments described above (Section 2.6.1). To optimize the assay, different annealing temperatures were tested. Amplification products of activin β_B and follistatin were sequenced at the sequencing platform of the University of Oslo. Gene specific primers for European eel FSHβ, LHβ, TSHβ (thyrotropin β subunit) GPα, GH (growth hormone) and ARP (acidic ribosomal phosphoprotein P0) were reported previously [4,61,79].

2.6.3. qPCR assays

Messenger RNA abundance was measured by qPCR using the Light Cycler 2.0 platform (Roche) with Light Cycler® FastStart DNA MasterPLUS SYBR Green I kit (Roche) according to the manufacturer's instructions. The primer final concentration in each case was 500 nM. The following conditions were used for the qPCR assays: an initial activation step of the Taq polymerase at 95 °C for 10 min, followed by 41–51 cycles of PCR: 95 °C for 15 s, 60 °C for 5 s and 72 °C for 10 s. Directly after the amplification phase, a melting curve analysis was carried out to confirm the presence of a single PCR product.

For tissue distribution study of activin β_B and follistatin, serial dilutions of whole brain were assayed in duplicate to determine the efficiency of the qPCR reaction. Serial dilutions of another tissue (retina) were also tested in order to assess the parallelism with the brain standard. Each individual sample of each tissue was then assayed in duplicate. Each PCR run contained a non-template control and a calibrator to adjust for assay to assay variations. Relative expression levels were calculated as previously described [79]. Transcript levels were normalized to total RNA content.

For cell culture experiments, serial dilutions were prepared from a pool of cDNA of all culture wells to be used as standards for FSHβ, LHβ, GPα, TSHβ, GH and ARP. Each well sample was then assayed in duplicate for each gene of interest. Transcript levels were normalized to the reference gene (ARP) RNA levels.

2.7. Statistical analysis

Data are presented as mean ± SEM. Statistical analyses were performed using Instat 3.0b (GraphPad, Inc., San Diego, CA). Comparison of means was performed using one-way ANOVA followed by Tukey multiple comparison test. Data were log-transformed when needed to meet test criteria. The level of significance was set to $P < 0.05$.

3. Results

3.1. Tissue distribution of activin β_B and follistatin expression in the European eel

Activin β_B and follistatin transcripts were present in a variety of tissues, as analyzed by qPCR (Fig. 1). Activin β_B was found to be expressed in all parts of the brain as well as in the pituitary. In the peripheral tissues, the highest expression levels were observed in the ovary and retina. Activin β_B expression was also measured in gill and adipose tissue while muscle and intestine contained very low mRNA levels. Transcripts were under detection level in the liver.

Follistatin was also found to be expressed in all parts of the brain and in the pituitary. In the peripheral tissues the highest expression levels were observed in the retina and adipose tissue, whereas low levels were measured in the other tissues investigated (liver, intestine, muscle, gill and ovary).

3.2. Time-dependent effects of activin B on eel FSHβ and LHβ expressions

Dispersed pituitary cells were treated or not (control) with recombinant human activin B (10^{-8} M) and cultures were harvested after 3 or 9 days of treatment. Activin B treatment induced a strong and time-dependent increase in FSHβ mRNA levels. After 3 days of treatment, a significant increase in FSHβ mRNA level was observed compared to control (4×, $P < 0.001$). After 9 days, a further increase was observed (13×, $P < 0.001$ as compared to control) (Fig. 2A).

Concerning LHβ expression a weak but not significant decrease was observed after 9 days of treatment (Fig. 2B). Depending on the experiment, activin induced no significant variation (for instance Figs. 2B and 7B) or a significant decrease (for instance Figs. 5 and 8) in basal LHβ expression.

As the effect of activin B on FSHβ expression was higher after 9 days compared to 3 days of treatment ($P < 0.01$), 9 days of treatment were chosen for the following experiments.

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Table 1
European eel gene specific primers for quantitative real-time PCR.

Gene	Primer	5′–3′ Sequence	Amplicons size (bp)	Accession Nos.	Refs.
LHβ	fw	TCA CCT CCT TGT TTC TGC TG	149	X61039	[4]
	rv	TAG CTT GGG TCC TTG GTG ATG			
FSHβ	fw	TCT CGC CAA CAT CTC CAT C	100	AY169722	[4]
	rv	AGA ATC CTG GGT GAA GCA CA			
TSHβ	fw	ACT TCT GCG TGG CCA TCA AC	95	X73493	[61]
	rv	GGA CCA CCA GGC GCT TCA CC			
Gpα	fw	TGC CGA CTC CAG GAG AAT AA	184	X61038	[4]
	rv	TGT TAT CCA GCC TTG TCA CC			
GH	fw	AAA TCG GAT GGG TAC TTG CTG	183	AY616666	[4]
	rv	ACC GTC ACC TAC ATC CTT CAT			
Activin β_B	fw	CAG GGA AAG TTC GGG AAG AC	100	GU269543	This study
	rv	GAC GAC GTG AGT TCA TCT GAT T			
Follistatin	fw	GAG AAG GGC TAC ATG CCT TG	92	FR839633	This study
	rv	TGC ACT GAA TGT CAT CAC AAG A			
ARP	fw	GTG CCA GCT CAG AAC AC G	107	AY763793	[79]
	rv	ACA TCG CTC AAG ACT TCA ATG G			

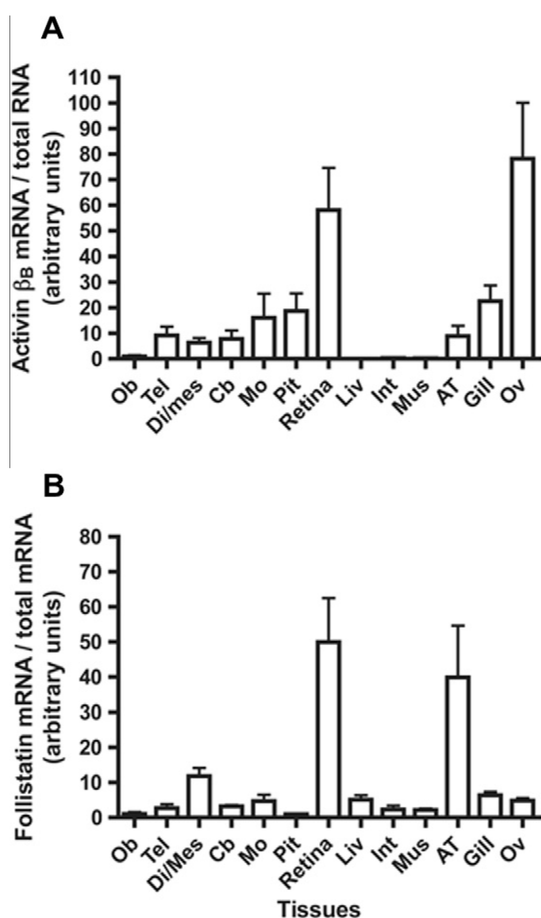

Fig. 1. Tissue distribution of European eel activin β_B and follistatin. Messenger RNA levels for activin β_B (A) and follistatin (B) were assayed by qPCR in various tissues: brain dissected in five parts: olfactory bulbs (Ob), telencephalon (Tel), di- and mesencephalon (Di/Mes), corpus cerebellum (Cb), and medulla oblongata (Mo); pituitary (Pit); retina; liver (Liv); intestine (Int); muscle (Mus); adipose tissue (AT); gill (Gill) and ovary (Ov). Data are normalized to total RNA. Means are given ± SEM ($n = 8$ eels).

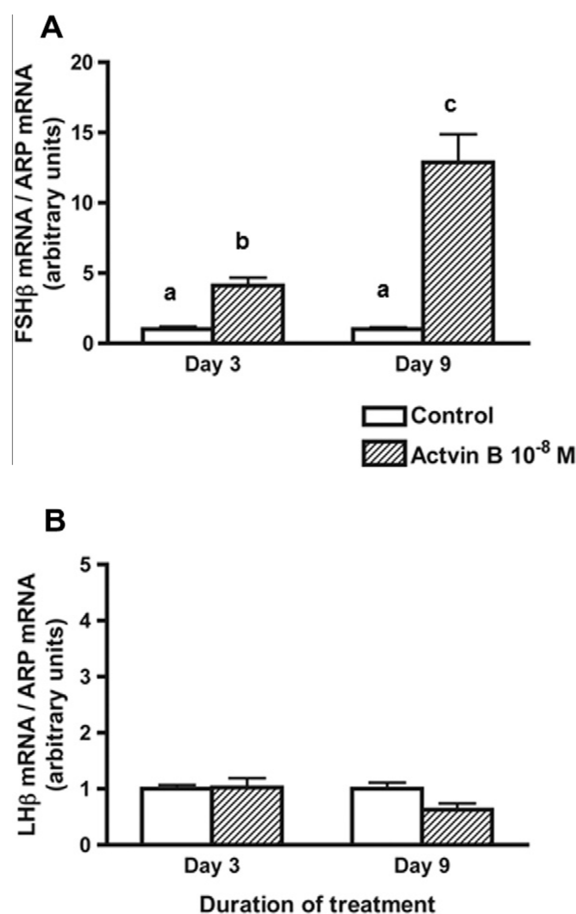

Fig. 2. Time-dependent effects of activin B on FSHβ (A) and LHβ (B) gene expression in eel pituitary cells. Cells were treated with 10^{-8} M recombinant human activin B and cultures were stopped at day 3 and day 9 to measure FSHβ and LHβ mRNA levels by qPCR. Data are normalized to eel ARP. Means are given ± SEM ($n = 5$ wells/group; 50,000 cells/well). Different letters indicate significant differences, ANOVA.

3.3. Dose-dependent effects of activin B on eel FSHβ and LHβ expressions

The effects of various concentrations (10^{-10}–10^{-7} M) of recombinant human activin B were analyzed after 9 days of treatment (Fig. 3). Activin B induced a dose-dependent stimulatory effect on FSHβ expression. The increase in FSHβ mRNA levels was significant at 10^{-10} M activin B (6×, $P < 0.01$, as compared to control). The highest stimulatory effect was observed at 10^{-7} M activin B (16×, $P < 0.001$, as compared to control) (Fig. 3).

In contrast, LHβ expression showed in the same experiment no dose-dependent changes (Fig. 3). Depending on the experiment, no dose-dependent variations (for instance Fig. 3) or a significant

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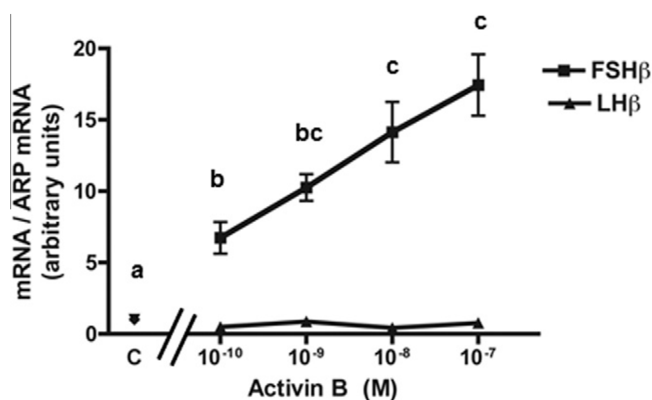

Fig. 3. Dose-dependent effects of activin B on FSHβ and LHβ gene expression in eel pituitary cells. Cells were treated for 9 days with various doses of recombinant human activin B and mRNA levels of FSHβ and LHβ subunits quantified by qPCR. Data are normalized to eel ARP. Means are given ± SEM ($n = 5$ wells/group; 50,000 cells/well). Different letters indicate significant differences, ANOVA.

and dose-dependent decrease (for instance Fig. 5) in basal LHβ expression have been observed.

3.4. Comparison of the effects of activin A and B on eel FSHβ expression

To investigate whether activin A had a similar stimulatory effect on FSHβ expression as activin B, pituitary cells were treated with various doses (10^{-11}–10^{-7} M) of either recombinant human activin A or activin B over 9 days (Fig. 4).

As in the previous experiment (Fig. 3) activin B was able to significantly and dose-dependently stimulate FSHβ expression (7× at 10^{-11} M, $P < 0.01$; 11× at 10^{-9} M, $P < 0.001$; 15.5× at 10^{-7} M, $P < 0.001$, as compared to control) (Fig. 4). Activin A had a similar dose-dependent effect as activin B on FSHβ mRNA levels (4× at 10^{-11} M, $P < 0.05$; 12× at 10^{-9} M, $P < 0.001$; 15× at 10^{-7} M, $P < 0.001$) (Fig. 4).

3.5. Effect of activin B on the gene expression of various eel pituitary hormones

To test the specificity of activin stimulatory action on FSHβ, pituitary cells were treated with different doses of recombinant human activin B (10^{-12}–10^{-8} M) over 9 days and mRNA levels of other pituitary hormones (GPα, TSHβ and GH) in addition to FSHβ and LHβ were assayed by qPCR (Fig. 5).

As in the above experiments, levels of FSHβ mRNA were dose-dependently increased by activin B (Fig. 5). In opposite, levels of LHβ mRNA were dose-dependently decreased (Fig. 5). In contrast, no significant effect of activin B was noticed on the expression of other pituitary hormones: TSHβ subunit, GPα subunit, and GH (Fig. 5).

3.6. Effects of follistatin alone or in combination with activin on eel FSHβ and LHβ expressions

The effects of various concentrations (10^{-9}–10^{-7} M) of recombinant human follistatin were tested over 9 days of pituitary cell culture. Follistatin alone had no significant effect at any doses on neither FSHβ nor LHβ gene expression (Fig. 6).

In order to test the potential antagonistic effect of follistatin on activin action, cells were treated for 9 days with 10^{-8} M activin B in the presence or not of 10^{-10} or 10^{-8} M follistatin (Fig. 7). As in the previous experiment (Fig. 6), follistatin alone had no effect on FSHβ expression (Fig. 7A). In contrast, follistatin was able to downregulate in a dose-dependent manner the stimulatory effect of 10^{-8} M activin B on FSHβ mRNA levels (0.6× at 10^{-10} M, $P < 0.05$ and 0.4× at 10^{-8} M follistatin, $P < 0.01$, as compared to activin B alone) (Fig. 7A).

The effect of activin B and follistatin alone or combined on LHβ expression was also investigated. Regardless of the treatment, no significant effects on LHβ mRNA levels were observed as compared to controls (Fig. 7B).

3.7. Effects of activin B and follistatin on LHβ expression by testosterone-treated eel pituitary cells

In the above experiments, activin B had a small or no significant inhibitory effect on basal LHβ expression. This weak and not reproducible effect could be due to the low initial basal levels of LHβ mRNA in silver eel pituitary cells. To test this hypothesis we chose to work on eel pituitary cells in which LHβ expression was increased by testosterone treatment [4,36,38]. Cells were co-treated with-testosterone and activin and/or follistatin.

Treatment with 10^{-8} M testosterone increased LHβ mRNA levels (6×, $P < 0.001$) (Fig. 8). Activin B was able to significantly decrease LHβ mRNA levels in testosterone-treated cells (0.4× at 10^{-10} M and 0.3× at 10^{-8} M activin B, $P < 0.001$, as compared to testosterone alone) (Fig. 8). In this experiment, 10^{-8} M activin B also showed a significant inhibitory effect on basal LHβ expression (0.3×, $P < 0.05$) (Fig. 8). While the effect of activin on basal LHβ mRNA levels was not always observed, its inhibitory effect on

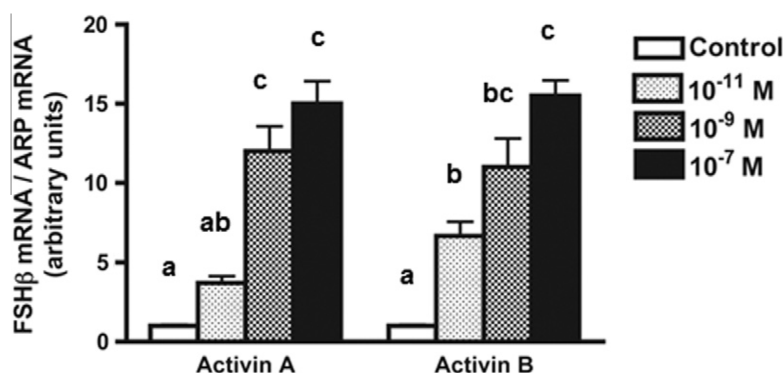

Fig. 4. Comparison of the effects of activin A and B on FSHβ gene expression in eel pituitary cells. Cells were treated for 9 days with various doses of recombinant human activin A or B and mRNA levels of FSHβ subunit quantified by qPCR. Data are normalized to eel ARP. Means are given ± SEM ($n = 5$ wells/group; 50,000 cells/well). Different letters indicate significant differences, ANOVA.

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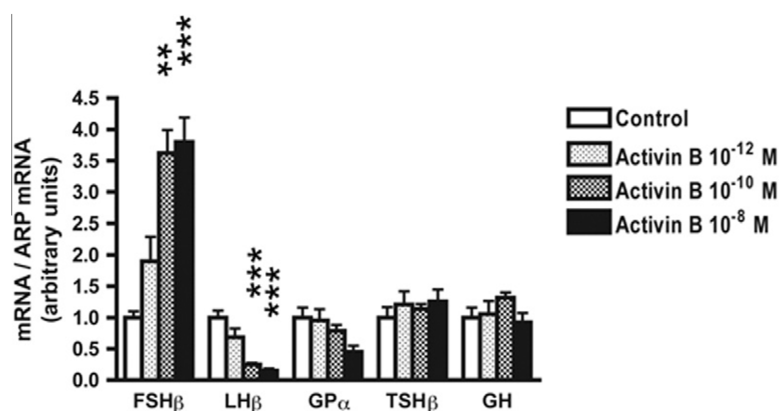

Fig. 5. Comparison of the effects of activin B on gene expression of various hormones in eel pituitary cells. Cells were treated for 9 days with various doses of recombinant human activin B and mRNA levels of FSHβ, LHβ, GPα, TSHβ subunits and GH quantified by qPCR. Data are normalized to eel ARP. Means are given ± SEM ($n = 5$ wells/group; 50,000 cells/well). $^{**}P < 0.01$, $^{***}P < 0.001$, versus respective controls, ANOVA.

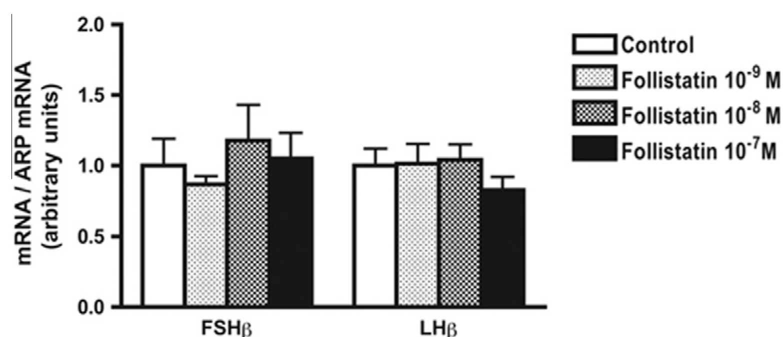

Fig. 6. Effects of follistatin on FSHβ and LHβ gene expression in eel pituitary cells. Cells were treated for 9 days with various doses of recombinant human follistatin and mRNA levels of FSHβ and LHβ subunits quantified by qPCR. Data are normalized to eel ARP. Means are given ± SEM ($n = 5$ wells/group; 50,000 cells/well).

testosterone stimulated LHβ mRNA levels was significant in every experiment performed (three independent experiments).

To test whether follistatin could antagonize the inhibitory effect of activin on LHβ expression, testosterone-treated cells were treated with recombinant human activin B and follistatin alone or in combination (Fig. 9).

As in previous experiments (Fig. 8), activin B significantly and dose-dependently inhibited testosterone-induced LHβ expression (0.5× at 10^{-10} M and 0.2× at 10^{-8} M activin B, $P < 0.05$ as compared to testosterone alone) (Fig. 9). Combined treatments with follistatin (10^{-8} M) reduced the inhibitory effect of activin. Testosterone-treated cells receiving the combined treatment with activin (10^{-10} M) and follistatin (10^{-8} M) reached LHβ mRNA levels not significantly different from cells treated with testosterone alone (Fig. 9).

4. Discussion

Silver eels remain at a prepubertal stage as long as the oceanic reproductive migration does not occur, a blockade that results from a deficient production of pituitary gonadotropins LH and FSH. Previous works from our group have shown that various factors such as sexual and cortico-steroids and insulin-like growth factors activate LH expression in European eel cultured pituitary cells, while they have little effects on FSH [4,36–38]. The present work aimed at investigating the potential role on eel FSH expression of the activin/follistatin system well known to control FSH in mammals.

Miura and co-workers demonstrated that testicular activin B is a major mediator of hormone-induced spermatogonial proliferation in the male Japanese eel and they were able, accordingly, to clone activin β_B cDNA from testis mRNA in males treated with human chorionic gonadotropin [53,54]. To our knowledge, no other investigation on eel activin has been performed. In the present study, we set up qPCR assays for European eel activin β_B and follistatin to study their tissue distribution, and we investigated the potential role of the activin/follistatin system on the direct regulation of eel gonadotropin subunit gene expression.

4.1. Wide tissue distribution of activin β_B and follistatin in the European eel

Considering the recent findings of multiple forms of activin β subunits in teleosts: two activin βA in zebrafish [19] and a new activin β_E in several teleosts (http://www.ensembl.org), future studies will aim at characterizing other members of the activin/follistatin system in European eel. In this study, we focused on activin β_B and the activin-binding protein, follistatin.

Analyses of tissue distribution by qPCR showed that activin β_B and follistatin are both expressed in the different parts of the eel brain. The presence of activin in the brain has also been observed in other teleosts, goldfish (activin β_A and β_B [26,28,29,80], rainbow trout (activin β_B [72] and grey mullet (activin β_A [57]. Similarly, follistatin has also been detected in the brain in some other teleosts, zebrafish embryo [6], goldfish [14] and seabream [24]. In mammals, activin and follistatin are also expressed in the brain where they may be involved in diverse functions, such as control

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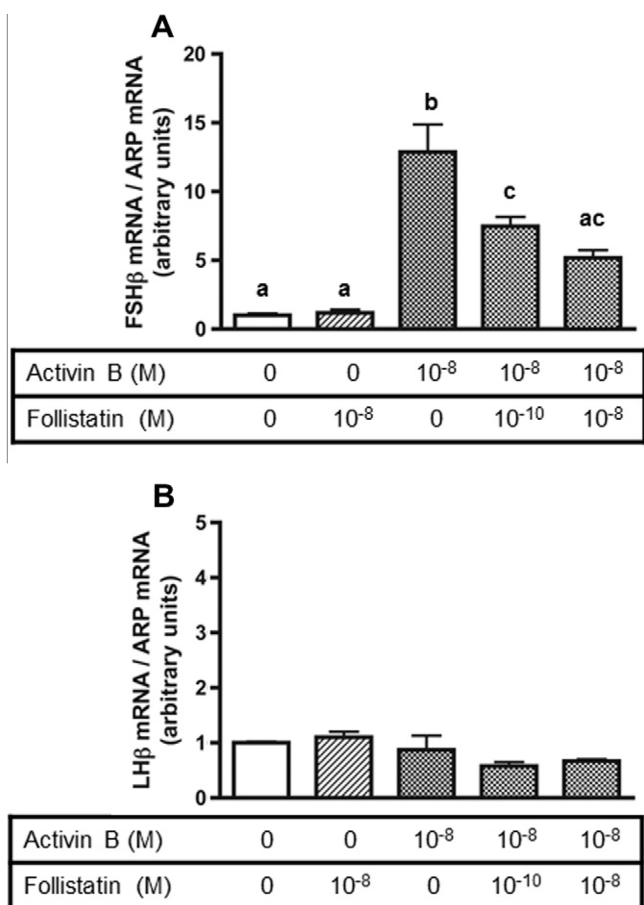

Fig. 7. Effects of combined treatments of activin B and follistatin on FSHβ and LHβ gene expression in eel pituitary cells. Cells were treated for 9 days with recombinant human activin B and follistatin, alone or in combination. Messenger RNA levels for FSHβ (A) and LHβ (B) subunits were quantified by qPCR. Data are normalized to eel ARP. Means are given ± SEM ($n = 5$ wells/group; 50,000 cells/well). Different letters indicate significant differences, ANOVA.

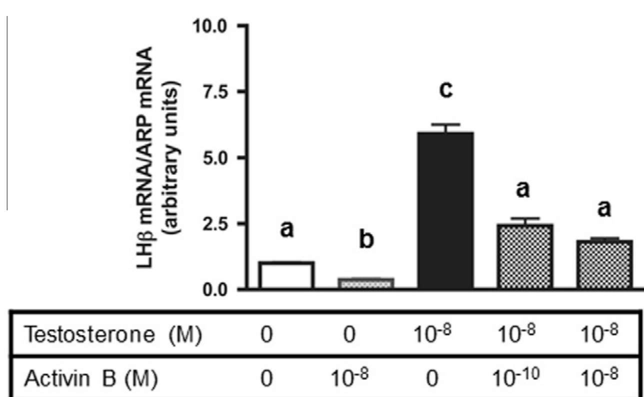

Fig. 8. Effects of activin B on LHβ gene expression in testosterone-treated eel pituitary cells. Cells were treated for 9 days with testosterone and recombinant human activin B (10^{-10} or 10^{-8} M), alone or in combination. Messenger RNA levels for LHβ subunits were quantified by qPCR. Data are normalized to eel ARP. Means are given ± SEM ($n = 5$ wells/group; 50,000 cells/well). Different letters indicate significant differences, ANOVA.

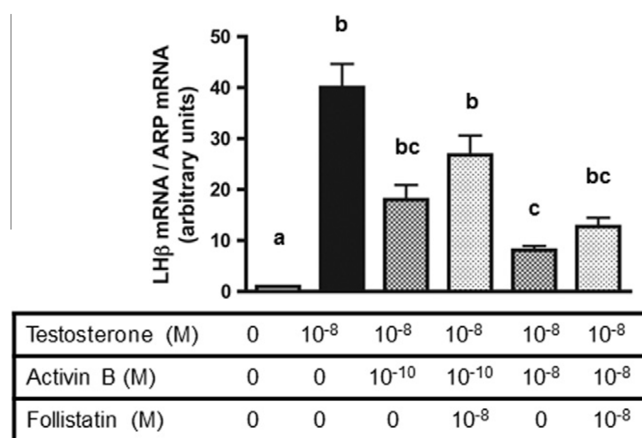

Fig. 9. Effects of combined treatments with activin B and follistatin on LHβ gene expression by testosterone-treated eel pituitary cells. Cells were treated for 9 days with testosterone, recombinant human activin B (10^{-10} or 10^{-8} M) and follistatin (10^{-8} M), alone or in combination. Messenger RNA levels for LHβ subunits were quantified by qPCR. Data are normalized to eel ARP. Means are given ± SEM ($n = 5$ wells/group; 50,000 cells/well). Different letters indicate significant differences, ANOVA.

of GnRH [47,48], modulation of anxiety behavior and adult neurogenesis [1].

Both activin β_B and follistatin transcripts were detected by qPCR in the eel pituitary. In goldfish, activin β_A and β_B subunits are expressed in the pituitary as revealed by immunocytochemical staining [26], Northern-blot analysis and RT-PCR [27,28,80]. In grey mullet pituitary, activin β_A immunoreactivity is observed in axon endings innervating somatotrope and gonadotrope cells, while activin β_B is detected in melanotrope cells close to gonadotropes [57]. Follistatin is expressed in the pituitary of goldfish [14], zebrafish [44], and seabream [24]. Activin receptors are found in the pituitary of goldfish [83], zebrafish [44], and grass carp [68]. Together, these data suggest local paracrine and/or autocrine roles for the activin/follistatin system in the teleost pituitary. Future studies on the co-expression of activin β_A and β_B subunit mRNA, as well as investigation at the protein level, would allow to further characterize which functional peptides are produced in the eel pituitary.

Activin is also produced in the pituitary in mammals suggesting a potential autocrine/paracrine function [8]. Moreover, works on rats indicate that activin β_A and β_B are expressed in pituitary gonadotropes [63]. In addition, the use of an activin B-specific antibody in cultured pituitary cells significantly suppressed the basal secretion of FSH, demonstrating that the endogenous pituitary activin could serve as a critical local factor for the control of gonadotropin expression and secretion [15]. In amphibians, immunocytochemical studies showed activin β_B immunoreactivity in gonadotropes (xenopus [74]; bullfrog [73]. In contrast, one study in the domestic fowl reported the absence of intrapituitary expression of activin subunits or follistatin using qPCR [46]. This suggests a lack of capacity for pituitary production of activin and follistatin in birds, which is different from other vertebrates.

Activin β_B transcripts were highly expressed in the European eel ovary as shown by qPCR. This result together with the previous one of Miura and collaborators in Japanese eel testis [53] confirm the presence of this subunit in gonads from both female and male eels. Activin β_B mRNA is also found in gonads in goldfish [26,29,80], rainbow trout [71], and grey mullet [57]. We also detected follistatin mRNA in the eel ovary. Follistatin recently has been cloned in some other teleosts and its gonadal expression demonstrated in goldfish [14], catfish [32], and seabream [24].

Concerning other peripheral organs, a remarkable finding from the present study is the high expression of both activin β_B and follistatin in the eel retina. To the best of our knowledge, this is the first evidence for such an expression in a teleost. The potential role of activins in the development of embryonic retina has been

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reported in other vertebrates (e.g. bird [7]; rodent [17]). The eel retina is known to be submitted to large changes even after the juvenile stage, during the silvering transformation and further during sexual maturation. A large increase in eye size as well as changes in retina photoreceptor pigments occur [3,60]. One may suggest that this plasticity could be related to the high expression of the activin/follistatin system.

Another striking finding is the high expression of follistatin and to a lesser degree activin β_B in the eel adipose tissue. This is the first report of their expression in adipose tissue in teleosts. Recent studies in human show that activin B participates in the control of adipocyte lipid metabolism in response to nutritional status [12,49,67]. In human and bovine, activin A inhibits the differentiation of preadipocytes while follistatin suppresses this effect of activin [35,84]. This opens new avenues for future investigations on the role of activin/follistatin system in teleost lipid metabolism, a key function for growth and reproduction.

4.2. Activin stimulates the expression of eel FSHβ subunit

Our results demonstrated that recombinant human activins A and B were able to induce a strong time- and dose-dependent stimulation of FSHβ expression by eel pituitary cells. These observations are in agreement with results obtained in other teleost groups. Studies in goldfish, a cyprinid, have demonstrated that recombinant goldfish activin B as well as recombinant human activin A upregulate FSHβ mRNA levels in goldfish pituitary cells [80,83]. In another cyprinid, zebrafish, a recent study demonstrated a stimulation of FSHβ expression in cultured pituitary cells treated with recombinant goldfish activin B [44]. In a salmonid, coho salmon, recombinant human activin A increases FSHβ mRNA levels in pituitary cells [16]. In a perciform, tilapia, preliminary studies have shown that cultured pituitary cells treated with recombinant human activin A also exhibited an increase in FSHβ subunit mRNA [81].

In an amphibian, the bullfrog, one study reports a positive effect of recombinant human activin B on FSH release by cultured pituitary cells [73]. In mammals, the stimulatory control of FSH expression by activin has been extensively investigated. The transcriptional activation effect of activin is mediated through activin response elements in the promoter of the FSHβ gene in human, ovine and rat [5,62,70,78]. Recent studies in teleosts indicate similar mechanisms. The use of the mouse LβT-2 cell line allowed to demonstrate that like mammalian FSHβ promoters, goldfish FSHβ promoter is stimulated by activin [43]. A binding site for Smad proteins, actors of the activin signaling pathway, has been recently characterized in the FSHβ gene promoter of Chinook salmon [78].

In the present study, we showed that recombinant human follistatin was able to significantly reduce the stimulatory effect of activin on FSHβ mRNA levels. This is in agreement with the well known antagonistic effect of follistatin in mammals, which binds to activin and the formation of this complex inhibits the interaction of activin with its receptors. In goldfish, recombinant human follistatin also inhibited the stimulatory effect of recombinant goldfish activin on FSHβ expression [83]. Recombinant goldfish follistatin was shown to have the same effect as human follistatin in this species [14]. All these data together with our present results in an ancient teleost, the eel, indicate that the positive regulation exerted by activin on FSH and its antagonism by follistatin would be an ancient regulatory mechanism common to the actinopterygian (teleosts) and sarcopterygian (tetrapods) lineages and largely conserved through vertebrate evolution.

4.3. Activin inhibits the expression of eel LHβ subunit

We observed that activin had only a weak inhibitory effect on LHβ expression, which could be due to the low basal LHβ levels in prepubertal silver eel [20]. To confirm the inhibitory effect of activin, we worked on testosterone-treated eel pituitary cells which present high LHβ mRNA levels as shown by our previous studies [4,38]. We demonstrated that recombinant human activin B induced a dose-dependent and reproducible inhibition of LHβ expression in these conditions. We also showed that follistatin was able to counteract the inhibitory effect of activin on LHβ.

An inhibitory effect of activin on LHβ expression has been previously described in cyprinids. In goldfish, treatment of pituitary cells by recombinant goldfish activin B or recombinant human activin A induces a decrease in LHβ mRNA levels [80,83]. Similar results were recently obtained in zebrafish after treatment of pituitary cells with recombinant goldfish activin B [44]. In contrast, such inhibitory control was not found in other teleosts investigated. In coho salmon, recombinant human activin A has no effect on LHβ mRNA levels in pituitary cells [16]. However, we may suggest as in our study that this lack of effect might result from the use of immature salmons with low LH levels [50]. A preliminary study in tilapia reports that recombinant human activin A increases LHβ subunit mRNA in pituitary cells [81]. The present finding of an inhibitory effect of activin on LHβ in the eel, a basal teleost, suggests that this could represent the ancestral role in teleost lineage. Additional studies in other species are needed to clarify possible species-and stage-variations in the role of activin in the regulation of LHβ expression among teleosts.

In the eel the opposite effects of activin on FSHβ and LHβ were limited to these pituitary gonadotropin subunits. No effect was recorded on the expression of TSHβ nor on the common glycoprotein subunit GPα nor on GH. This indicates a specific action of activin in regulating the gonadotropin β subunits in the eel. In zebrafish recombinant goldfish activin B downregulates both LHβ and GH expression [44]. In contrast, it should be noted that in another cyprinid, the goldfish, porcine activin stimulates GH release [28].

In bullfrog, one study reports that recombinant human activin B stimulates both FSH and LH secretion by pituitary cells *in vitro* [73] while another study shows that recombinant human activin A stimulates the release of FSH, GH, and PRL but not that of LH [41]. In mammals, activin does not exert any direct control on LH but modulates its expression and release via indirect mechanisms. For instance, activin regulates GnRH synthesis and release by hypothalamic explants and neuronal cell lines [11,31,47]. In the eel brain, we detected activin expression in telencephalon and di/mesencephalon, two regions where GnRH neurons are located [56]. In mammals, activin also enhances GnRH stimulatory effect on LH by increasing pituitary GnRH receptor transcription [10,23,59]. Such indirect mechanisms have not yet been investigated in other vertebrates.

5. Conclusions

In conclusion, we have shown the presence of an activin/follistatin system in the eel, its wide tissue distribution and its involvement in the regulation of FSH expression. Activin is the first major stimulator of FSH expression evidenced so far in the eel. The stimulatory control of FSH by activin and its antagonism by follistatin may represent an ancestral regulatory mechanism that appeared in a common ancestor to sarcopterygians and actinopterygians, and that has largely been conserved through osteichthyan evolution. To our knowledge, no activin has been described yet in chondrichthyes or petromyzontides, but follistatin has been characterized in the lamprey [34], suggesting the possible existence of an activin/follistatin system earlier in vertebrate evolution. We found that activin exerts an opposite effect on eel gonadotropins by stimulating FSHβ but inhibiting LHβ gene expression. Such a direct inhibition of LHβ expression is also

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evidenced in cyprinids but not in some other teleosts nor in amphibians or mammals. This suggests that this inhibitory control of LH may have emerged in basal teleosts but highly varied throughout evolution.

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