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Responsiveness of pituitary to galanin throughout the reproductive cycle of male European sea bass (*Dicentrarchus labrax*)

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Abbreviations: AbFsh, antisera against follicle stimulating hormone; AbLh, antisera against luteinizing hormone; AC, adenylyl cyclase; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; DM, dispersion medium; *ef1α*, elongation factor 1α; ELISA, enzyme-linked immunosorbent assays; FBA, fetal bovine serum; Fsh, follicle stimulating hormone; Fsk, forskolin; Gal, galanin; Galr/*galr,* galanin receptor; Gal-ir, galanin immunoreactivity; Gmap, galanin message associated peptide; GnRH, gonadotropin-releasing hormone; GSI, gonadosomatic index; *kiss,* kisspeptins; Lh, luteinizing hormone; PLC, phospholipase C; qPCR, quantitative polymerase chain reaction; RNA, sGnrh, salmon gonadotropin-releasing hormone. Protein and gene nomenclature followed that recommended by genenames.org and used for fish at http://zfin.org/; in this abbreviation list, for each case protein abbreviation is presented first followed by the corresponding gene abbreviation.

Abstract

The neuropeptide galanin (Gal) is a putative factor regulating puberty onset and reproduction through its actions on the pituitary. The present study investigated the pituitary responsiveness to galanin and the patterns of galanin receptors (Galrs) expression throughout the reproductive cycle of two years old male European sea bass (*Dicentrarchus labrax*), an important aquaculture species. Quantitative analysis of pituitary and hypothalamus transcript expression of four *galr* subtypes revealed differential regulation according to the testicular developmental stage, with an overall decrease in expression from the immature stage to the mid-recrudescence stage. Incubation of pituitary cells with mammalian 1-29 Gal peptide induced significant changes in cAMP concentration, with sensitivities that varied according to the testicular development stages. Furthermore 1-29 Gal was able to stimulate both follicle stimulating hormone (Fsh) and luteinizing hormone (Lh) release from pituitary cell suspensions. The magnitude of the effects and effective concentrations varied according to reproductive stage, with generalized induction of Fsh and Lh release in animals sampled in January (full spermiation). The differential expression of *galrs* in pituitary and hypothalamus across the reproductive season, together with the differential effects of Gal on gonadotropins release *in vitro* strongly suggests the involvement of the galaninergic system in the regulation the hypothalamus-pituitary-gonad axis of male sea bass. This is to our knowledge the first clear evidence for the involvement of galanin in the regulation of reproduction in non-mammalian vertebrates.

Keywords: European sea bass; galanin; gonadotropins; reproduction; hypothalamus; pituitary

1. Introduction

Galanin (Gal) is a multi-functional neuropeptide widely expressed in the central and peripheral nervous system across the vertebrates (Gai et al., 1990; Mensah et al., 2010). The *gal* gene structure is highly conserved and the preprogalanin mRNA precursor is composed of distinct regions encoding the signal peptide, a well-conserved 29 amino acid mature peptide (30 in human) and a galanin message associated peptide (Gmap) (Kofler et al., 1996; Mensah et al., 2010). Variant transcript forms resulting from alternative spicing have been reported in non-mammalian vertebrates, including one fish species (goldfish, *Carassius auratus*), but their biological significance is still unknown (Mensah et al., 2010; Unniappan et al., 2003). In mammals, Gal functions appear to be mediated by three specific G-protein coupled receptors (Galr1, Galr2 and Galr3), which vary in their distribution, G-protein coupling and signalling mechanisms (reviewed by Gundlach, 2002; Webling et al., 2012). Galrs have not yet been functionally characterized in non-mammalian vertebrates, but in teleost fishes such as the European sea bass (*Dicentrarchus labrax*), henceforth sea bass, duplicate paralogs of *galr1* and *galr2* (*galr1a, galr1b, galr2a* and *galr2b*) exist while *galr3* orthologues have not been identified (Martins et al., 2014).

In mammals, galanin has been implicated in several physiological functions including feeding behaviour, nociception, memory and cognition, mood, nerve repair, gut motility and reproduction (Crawley, 1999; Fang et al., 2015; Hohmann et al., 2003; Rustay et al., 2005). Galanin is strongly expressed in the hypothalamus and anterior pituitary and in rat (*Rattus norvegicus*) Gal immunoreactivity (Gal-ir) has been detected in nearly 50% of the neurons of the hypothalamic-neurohypophyseal system (Arai et al., 1990). Gal is believed to participate in the neuroendocrine control of gonadal functions by stimulating the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus, where Gal co-localizes in GnRH neurons and has sex steroid and seasonal regulation (Dudas and Merchenthaler, 2004; Merchenthaler et al., 1991; Pandit and Saxena, 2010; Rossmanith et al., 1996). In the pituitary it stimulates gonadotropin secretion or modulates GnRH-

stimulated gonadotropin secretion (e.g. Baratta et al., 1997; Pandit and Saxena, 2010; Splett et al., 2003; Todd et al., 1998). It also stimulates the pituitary secretion of other hormones such as prolactin and growth hormone (e.g. Baratta et al., 1997; Todd et al., 1998; Wynick et al., 1998).

Gal regulation of the mammalian hypothalamo-neurohypophyseal system may become more relevant around puberty (Fang et al., 2015), the developmental period where immature animals activate the pulsatile secretion of hypothalamic GnRH that stimulates pituitary gonadotropin secretion and subsequent sex steroid production and gametogenesis. For example, Gal-ir and mRNA expression in GnRH neurons increased significantly during puberty in both male and female rats and humans (Celi et al., 2005; Planas et al., 1994; Rossmanith et al., 1994). Given its roles regulating feeding (being orexigenic, appetite stimulating) and reproduction, Gal was recently proposed as an integrator between energy metabolism and reproduction (reviewed by Celik et al., 2015; Fang et al., 2015).

In non-mammalian vertebrates, however, the functions of Gal are still poorly understood (Mensah et al., 2010). In goldfish and tench (*Tinca tinca*) mammalian Gal stimulated food intake *in vivo* (de Pedro et al., 1995; Guijarro et al., 1999; Volkoff and Peter, 2001) and a possible role in gut motility has been suggested through localization and *in vitro* studies (Karila et al., 1993). Possible functions in the control of reproduction have also been suggested on the basis of the sex dimorphic patterns of Gal-ir in the brain and pituitary of some teleost species (e.g. Cornbrooks and Parsons, 1991; Rao et al., 1996; Rodriguez et al., 2003), and by the observation of Gal seasonal variations and regulation by sex steroids in the eel (*Anguilla anguilla*) brain (Olivereau and Olivereau, 1991a; Olivereau and Olivereau, 1991b). In sea bass, Gal-ir cell bodies are located in the anterior and posterior hypothalamus and Gal-ir nerve fibres penetrate the pituitary *pars distalis*, in close contact with prolactin, growth hormone and gonadotropin secreting cells (Batten et al., 1990; Moons et al., 1989). Gal binding sites have been detected in several areas of the sea bass brain (Moons et al., 1991) and the four *galr* transcripts are expressed in the anterior and mid-brain of male and female (Martins et al., 2014). Interestingly, the *galr1b* transcript was up regulated in the pre-pubertal sea bass brain by an artificial photoperiod

regimen (“accelerating” or “compressed” photoperiod) shown to advance gametogenesis (Martins et al., 2015; Carrillo et al., 2015; Rodriguez et al., 2001) and in pre-pubertal testes by an androgen treatment (Martins et al., 2014). Altogether, these evidences support the hypothesis of a possible involvement of the galaninergic system in the regulation of fish reproductive function, in particular in the European sea bass. The sea bass is an important species for European fisheries and aquaculture, affected by a high rate of slow growing precocious males under intensive culture (Taranger et al., 2010), and is a useful fish model for basic and applied research in reproduction, with the genome available and where a vast body of information exists on the hormonal and developmental changes that accompany the first and consecutive reproductive seasons in males and females (Alvarado et al., 2013; Carrillo et al., 2015; Mazon et al., 2015).

To test the hypothesis of a possible Gal involvement in male sea bass reproduction we analyzed the seasonal patterns of *galr* expression in the hypothalamus and pituitary of male sea bass, and the *in vitro* bio-activity of Gal on the release of follicle stimulating hormone (Fsh) and luteinizing hormone (Lh) and on cyclic adenosine monophosphate (cAMP) production, using pituitary cell suspensions.

2. Materials and Methods

2.1 Animals and sampling

Animal maintenance and experimentation was carried out in certified experimental facilities and followed national legislation of Portugal (DL 113/2013) under a 'group-1' license by the Veterinary General Directorate, Ministry of Agriculture, Rural Development and Fisheries of Portugal. Two-years old male sea bass used in pituitary cell culture studies were obtained from AtlantikFish (Castro Marim, Portugal). They were maintained at the experimental station of Ramalhete in 1000 L tanks with continuously running natural seawater, under natural photoperiod (between 10:14 hours light-dark, LD, in winter, and 15:9 in summer) and natural temperature (between 11ºC in winter and 25ºC in summer). Fish were fed with commercial pellets (Sparos, Portugal). For pituitaries sampling for cell

culture, fish were anaesthetized on iced water and sacrificed by decapitation. Pituitaries were immediately placed in dispersion medium (see below). Small pieces of testis were fixed in 4% formaldehyde for hematoxylin-eosin histological staging of developmental stages following the classification previously described in this species (Begtashi et al., 2004): Stage I, immature; Stage II, early recrudescence; Stage III, mid recrudescence; Stage IV, late recrudescence; Stage V, full spermiating testes and Stage VI, resting. The gonadosomatic index (GSI) was calculated as gonad mass/body mass x 100.

2.2 Quantitative RT-PCR (qPCR)

Gene expression of the four sea bass *galr* (Martins et al., 2014) was quantified in male sea bass pituitary and hypothalamus across the reproductive cycle using RNA samples from the study of Alvarado *et al*. (Alvarado et al., 2013). Briefly, 2 year-old males (probably first-time spawning) were sampled from August to late April, covering the period from the beginning of spermatogenesis to post-spermiation. Fish were killed with an overdose of anesthetic, tissues were collected, snap frozen and stored at -80ºC until RNA extraction.

Total RNA was extracted using the Maxwell 16 LEV simply RNA purification kit (Promega), including a DNAse treatment. RNA quantity and quality were quantified with a NanoDrop 1000 (Thermo Fisher Scientific, USA) and cDNA synthesis was carried out in 20µl reactions containing 500 ng of RNA and 200 ng of random hexamers. Transcript levels were analyzed by qPCR using the relative standard curve method and the EvaGreen chemistry (1x Sso Fast EvaGreen Supermix, Bio-Rad), as previous described (Martins et al., 2014). Transcript copy number was calculated as described in (Pinto et al., 2013) and expression profiles were normalized by dividing copy number of the target transcript by the copy number of the reference gene, elongation factor 1α (*ef1α*) (Alvarado et al., 2013).

2.3 Dispersed pituitary cell culture

Pituitary primary cell suspensions were prepared throughout the reproductive cycle with two years-old male sea bass: immature (September, n = 33; body mass 430.1 ± 15.65 g; GSI = 0.076 ± 0.004 %);

recrudescent (November, n = 31; body mass = 513.6 ± 17.36 g; GSI = 0.164 ± 0.027 %) and spermiating (January; body mass = 588.4 ± 25.27 g; GSI = 2.596 ± 0,179 %; n = 32). All individuals used in pituitary cell culture in September were in testicular development stage I (histology analyzed *a posteriori*). In November 40% of the fish were in stage I, 36% in stage II, 8% in stage III, 12% stage in IV and 4% in stage V. In January all individuals were spermiating (stage V).

The dispersed pituitary cell suspensions were prepared as previously described (Peyon et al., 2003) with minor modifications. Briefly, in each experiment the pituitaries from 5 male sea bass were pooled and placed in ice-cold dispersion medium, DM (L-15 with Hank's balanced salts, 25 mM Hepes, 0.5 % bovine serum albumin (BSA), 1% penicillin–streptomycin and 0.1 mg/ml gentamicin, pH 7.4). Excised pituitaries were washed three times in DM, diced and exposed sequentially to trypsin (type II, 25 mg/10 ml, 45 min) and DNAse II (0.1 mg/10 ml, 10 min) digestions. Fragments were mechanically dispersed in DM by suction and extrusion using plastic transfer pipettes. Dispersed cells were filtered through a nylon mesh (100 µm pore size), harvested by centrifugation at 200*g* for 10 min and reconstituted in 5 ml FBS (fetal bovine serum)-free cultivation medium (L-15 with 0.1% BSA, 1% penicillin–streptomycin, and 0.1 mg/ml gentamicin, pH 7.4). Cell yield and viability was estimated using trypan blue staining.

2.4 In vitro assays

cAMP production was assessed in dispersed pituitary cells incubated with rat/mouse 1-29 galanin (Tocris, UK) and / or with the ubiquitous adenylyl cyclase (AC) activator forskolin (Fsk). Prior to the assay, cells were re-suspended in FBS free cultivation medium containing 1 mM of 3-isobutyl-1-methylxantine (IBMX, Sigma) and incubated for 5 min at 20°C. Cells were cultured in white 384 well small volume HiBase Polystyrene microplates (Greiner, Germany) at 15,000 cells/well. Incubations were carried in FBS free culture medium containing Gal alone (rat/mouse 1-29 Gal, 10-fold dilutions between 1 pM - 100 nM) or 1 pM – 10 nM Gal combined with 200 nM Fsk. Cells were incubated for 30 min at 20°C and cAMP production measured by the cAMP dynamic 2 kit (Cisbio, France)

according to the manufacturer's protocol, using a Biotek Synergy 4 plate reader (Biotek, USA). Data were normalized following the manufacturer's recommendations. Results were presented as the percentage of cAMP production relative to the stimulation with 200nM Fsk.

To evaluate the effects of Gal on Fsh and Lh release, the same pituitary cell suspensions used in cAMP assays were cultured overnight at 20ºC at a density of 2.5 x 10^5 cells/well, in 96-well culture plates containing culture medium with 10% FBS. Medium was replaced by 0.1 ml per well of serum-free culture medium containing freshly diluted Gal (1pM, 100 pM or 10 nM rat/mouse 1-29 Gal), incubated for 1 h and the collected culture medium stored at -80°C until analysis. A positive control incubated with salmon gonadotropin-releasing hormone (sGnrh; Sigma-Aldrich, Portugal) was also carried out and assayed for stimulation of Lh/Fsh release (Forniés, 2003). Fsh and Lh levels were measured in the culture media using specific competitive enzyme-linked immunosorbent assays (ELISA) as previously described using homologous antisera: AbFshβ-2 and AbLhβ with the recombinant Fsh and Lh heterodimers as standards (Moles et al., 2012) (Mateos et al., 2006). Both cAMP and gonadotropins studies were repeated six times with different pituitary cell preparations from pools of 5 pituitaries *per* assay, performed in triplicate.

2.5 Data representation and Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical differences between group means of Fsh, Lh or cAMP levels in treated versus control groups was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test. Differences in relative expression levels of sea bass *galr* between testicular developmental stages were analyzed by one-way ANOVA on log2-transformed data, followed by the Tukey's test. Statistical significance was set at $p<0.05$.

Pearson correlations were calculated on log2-tranformed *galr* expression levels. The error was set at 0.05 and divided by the number of comparisons and only *p* values lower than the corrected value were considered significant (Bonferroni correction).

3. Results

3.1 Changes in hypothalamic and pituitary galr mRNA levels during gametogenesis

The *galr* expression profiles were quantified in the hypothalamus and pituitary of 2 years-old male sea bass sampled between the end of August and mid-May, grouped according to their testicular development from stages I to VI (Fig. 1). In general, *galr2* subtypes were particularly highly expressed in the hypothalamus compared to the pituitary and the *galr1* subtypes. There were no significant differences between the expression levels of *galr1a, galr1b* and *galr2b* at different testicular stages. The expression of hypothalamic *galr2a* was highest at stage I and decreased significantly thereafter from stages II to VI (Fig. 1).

In contrast to *galr2* subtypes, *galr1* subtypes were more highly expressed in the pituitary (Fig. 2). Pituitary *galr1a* expression decreased from testicular stage I to stage III (mid-recrudescence), reaching minimal values at stage III and significantly increasing at stages V (spermiating) and VI (resting). Pituitary *galr1b* and *galr2b* levels were higher in pituitaries of immature animals (stage I), showing a significant decline between stages I and III and then stabilizing at low levels at more mature stages (Fig. 2). The expression of these two genes was significantly positively correlated ($R = 0.687$ $p < 0.001$, n=27). In contrast, the pituitary expression of *Galr2a* was always low.

3.2 In vitro effects of Gal on pituitary cAMP production

To provide an additional assessment of the pituitary responsiveness to galanin, the global production of cAMP to rat/mouse 1-29 Gal was measured at different points of the reproductive cycle of two years old male sea bass. No significant effects of Gal alone on cAMP production were detected at any of the sampling points (not shown). Only when cells were incubated simultaneously with Gal and forskolin was there a statistically significant decrease in cAMP production compared to that of cells incubated with Fsk alone (Fig.3), with the sensitivity to Gal varying seasonally. While in September Gal was effective in decreasing global cAMP levels by approx. 50% at 10 pM or above, in November significant decreases were detected only at 100 pM and 10 nM. In January, all tested concentrations

significantly decreased cAMP production in Fsk stimulated cells, with higher magnitude for 1 pM Gal (approx. 50% decrease) while for higher concentrations the decrease was smaller (20-30% compared to Fsk alone).

3.3 In vitro effects of Gal on Fsh/Lh release by pituitary cells

In September the basal *in vitro* release of Fsh in control pituitaries was relatively low (around 50 ng mL^{-1}) (Fig. 4). Gal (1pM) stimulated Fsh release (P<0.001) as did the sGnrh positive control at 10nM (P<0.05). No stimulation of Fsh release was detected in cells incubated with 100pM or 10nM Gal. In November the basal Fsh release in control pituitaries was slightly higher than in September (around 80 ng mL^{-1}) and no stimulation of Fsh release was obtained with Gal or sGnrh (Fig. 4). In January, basal release of Fsh was much higher (around 260 ng mL^{-1}) and there was statistically significant stimulation of Fsh release with 1pM, 100pM (P<0.001), 10nM (P<0.001) of Gal and with 10 nM of sGnrh (P<0.001).

Lh basal release was lower in September and in November (around 200 ng mL^{-1}) and Gal or sGnrh did not stimulate Lh release at any of the tested concentrations (Fig. 5). In January, basal release of Lh was substantially higher (around 1500 ng mL^{-1}) and Gal at 1 pM, 100 pM and 10 nM (P<0.001) and sGnrh at 10nM (P<0.0001) stimulated release of Lh.

4. Discussion

In this study, we showed evidence supporting the involvement of the galaninergic system in central regulation of reproductive functions in male sea bass. We showed stimulation of *in vitro* pituitary release of Fsh and Lh by Gal in two-year-old male sea bass and that the response was stage/season dependent. Similarly, Gal receptor expression levels in brain and pituitary varied according to testicular developmental stages.

An initial assessment of male sea bass galinergic system in the hypothalamus and pituitary showed that the four previously described *galr* subtypes (Martins et al., 2014) expressed throughout the whole

reproductive cycle. There was a differential expression between the hypothalamus and the pituitary, with apparent preponderance of *galr1* subtypes in the pituitary, and of *galr2* subtypes in the hypothalamus. These results are in line with early descriptions of Gal-binding sites in sea bass and other species (e.g. Holmqvist and Carlberg, 1992; Masini et al., 2006; Moons et al., 1991) and now provide more detailed information. In addition, Gal receptor expression was differentially regulated throughout the reproductive cycle. Hypothalamic *galr2a* was strongly downregulated at stage II compared to the immature stage, and progressively restored levels as spermatogenesis progressed. In the pituitary the other three receptors showed higher levels in immature fish, which were significantly down regulated at mid-recrudescence (stage III) and remained low during the rest of the cycle, for the correlated genes *galr1b* and *galr2b,* or increased again at spermiation, for *galr1a*. These results support that galanin can have direct actions in pituitary and hypothalamus, probably acting on the specific Galrs, and identified patterns of *galr* expression according to testicular stages reveals a developmental regulation of galanin responsiveness along the reproductive season. The expression patterns of pituitary *galr1b* and *galr2b* resembles *kiss1* and its receptor *gpr54-2b* (and hypothalamic *kiss2* and *gpr54-2b*) in the same panel of fish (Alvarado et al., 2013). As kisspeptins have been established as key factors in sea bass reproduction (Carrillo et al., 2015; Espigares et al., 2015a; Espigares et al., 2015b), namely regulating the pituitary secretion of gonadotropins, this may indicate that the two peptide systems are involved in the same or similar processes.

In mammals, Gal regulates growth hormone, prolactin and Lh secretion in male and female pituitary cells (e.g. Baratta et al., 1997; Elsaesser, 2001; Fang et al., 2015; Lopez et al., 1993; Pandit and Saxena, 2010; Scheffen et al., 2003; Wynick et al., 1998). In fish, Gal-ir was detected the pituitary in close contact with prolactin, growth hormone and Fsh/Lh-secreting cells (e.g. Anglade et al., 1994; Batten et al., 1990; Mensah et al., 2010; Moons et al., 1989; Power et al., 1996). Based on this, we focused on the secretion of gonadotropins, essential regulators of reproduction.

Gal stimulated the release of both Lh and Fsh from pituitary cells of male sea bass at different stages of testicular maturation. The different effects and sensitivities to Gal observed between immature and spermiating fish could result from differences in the relative levels of different *galrs* (as discussed below) or on the availability of stored hormones. Our results showed higher basal levels of Fsh and Lh in January, when more substantial effects of Gal were detected. Furthermore, the effects of sGnrh (a potent stimulator of gonadotropins release in sea bass (Forniés, 2003)) paralleled that of Gal, stimulating Fsh release in immature animals and Fsh and Lh in spermiating fish. In agreement with this, a small peak in plasma Fsh was previously detected around October-November in 2-years-old male sea bass while the most prominent peaks in plasma and pituitary gonadotropins have been observed in January-February in spermiating fish, when pituitary *fshβ* and *lhβ* levels are also at their highest levels (Alvarado et al., 2015; Mateos et al., 2003).

Our results confirm that Gal is able to regulate male sea bass reproduction through direct regulation of pituitary release of gonadrotropins at particular reproductive stages, which could further potentiate their reported effects regulating gonadal functions (Mazon et al., 2015). Gal effects on Fsh but not Lh release in pituitary cells from fish at the beginning of the reproductive cycle, stimulating release of both forms at later stages, are in agreement with previous studies suggesting that Fsh plays a role in sea bass during early-mid phases of spermatogenesis and in steroidogenesis, whereas Lh is involved in late reproductive events such as spermiation (Mazon et al., 2015; Moles et al., 2011). Our internal control quantifications of total Fsh/Lh contents of *in vitro* pituitary cells before incubation with Gal demonstrated no significant differences compared to the total gonadotropin levels detected in the medium in the groups where maximum release levels were reached (data not shown). As such, this suggests that detected Gal-induced increases in culture media gonadotropin levels could more likely be due to hormone release rather than *de novo* synthesis.

It remains to be established if, like in mammals, Gal also regulates pituitary gonadotropin secretion indirectly by modulating hypothalamic Gnrh secretion or pituitary Gnrh-regulated gonadotropin secretion (reviewed in Fang et al., 2015), to confirm Gal pituitary actions *in vivo* and investigate actions at other points of the hypothalamus-pituitary-gonads axis. Interestingly, in several studies in mammals pituitary Lh secretion could not be directly stimulated by Gal alone (Pandit and Saxena, 2010; Scheffen et al., 2003; Splett et al., 2003), only when combined with Gnrh, and Gal appears not to influence Fsh secretion (Arvat et al., 1995; Lopez et al., 1993; Sanchez-Criado et al., 2001). This suggest possible differences in Gal reproductive functions between mammals and fish. In addition, in mammals, the well-established modulation of Gnrh and Lh secretion by Gal appears to be dependent on the levels of sex steroids (Fang et al., 2015; Rossmanith et al., 1994; Scheffen et al., 2003). In the present study, highest Gal effects on Lh, Fsh and cAMP were observed in spermiating fish, after plasma androgens have reached their highest levels (Alvarado et al., 2015; Rocha et al., 2009), but some effects were also detected in fish with immature testes and further studies will be required to evaluate possible dependence of Gal actions on sex steroid levels.

In mammals, Gal family members have been proposed as integrators between energy metabolism and reproduction (reviewed in Celik et al., 2015; Fang et al., 2015). Currently there are evidences for conserved Gal functions in fish, namely stimulating food intake (de Pedro et al., 1995; Volkoff and Peter, 2001). The current work provides strong evidence for Gal direct actions on neuroendocrine control of reproduction in male sea bass. Thus, in fish, galanin may be one of the factors linking metabolism and reproduction and we have previously suggested that Gal could play a role regulating the onset of puberty (Martins et al., 2015; Martins et al., 2014). The present study used 2-year-old male sea bass, which would be in their first reproductive season; however, since in captivity male sea bass present a high incidence of precocious puberty, usually affecting the larger fish (Carrillo et al., 2015; Carrillo et al., 2009), some of the assayed fish may have matured in their first year. Thus, the present results are evidence of Gal effects on reproduction in male sea bass and future studies will focus on

early events of maturation in both precocious and non-precocious males to test possible roles for Gal as an integrator of external (e.g. photoperiod) and internal (e.g. growth or adiposity status) signals at puberty onset.

Our results also provide a preliminary insight into the mechanisms involved in Gal actions in male sea bass brain and pituitary. Relative *galr* expression levels and regulation may be informative of their contribution in mediating Gal effects detected at each sampling point and suggest a major role for *galr1b* at immature stages and *galr1a* in spermiating fish. Individual *galr* localization studies will be required to evaluate in which cell types are they expressed (e.g. if they are expressed in gonadotropes, responsible for Lh and Fsh secretion), if they co-localize and if they follow the same seasonal regulation in specific cell types. In addition, the significant alterations in cAMP production detected in pituitary cell suspensions provide a first evidence that this is one of the signaling pathways used by Gal in fish pituitaries. This is consistent with the detected high levels of *galr1* subtypes and with the information available from mammals, where Galr1 is strongly associated with the inhibition of the adenylate cyclase pathway (AC) while Galr2 stimulates phospholipase C (PLC) and is weakly coupled to the AC pathway (Wang et al., 1998; Webling et al., 2012). However, the detected responses do not necessarily indicate that Gal effects increasing Lh and Fsh secretion are mediated through inhibition of the AC pathway in the gonadotropes, as cAMP was measured in primary pituitary cell suspensions and the global levels may derive from signaling through different receptors and in multiple cell types. Although it has been previously suggested that activation of the AC pathway is one of the pathways used by Gnrh stimulation of Lh release in fish, the mechanisms involved remain largely unexplored (Rebers et al., 2000; Zohar et al., 2010). The present results, interestingly show that the sensitivity to Gal towards Fsh/Lh release generally paralleled the cAMP responses during the reproductive cycle, with highest sensitivity in spermiating followed by immature fish. Further studies will be required to characterize the signaling mechanisms used by each isolated fish Galr and the possible involvement of the AC, PLC or other pathways mediating Gal effects on the pituitary gonadotropin release.

5. Conclusions

Our results provide strong evidence for differential receptor expression and differential galanin effects on Fsh and Lh release in 2-years old male sea bass, depending on their testicular development stages. They support the initial hypothesis that Gal is able to directly regulate pituitary gonadotropin release and suggest that these effects may be mediated, at least in part, by Galr1 subtypes and possibly involving the AC signaling pathway. Within the lack of knowledge on the importance and function of Gal in non-mammalians vertebrates, this is the first clear evidence of the involvement of Gal regulating reproduction in sea bass and probably also in other fish species.

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Conflicts of interest

None.

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Figures

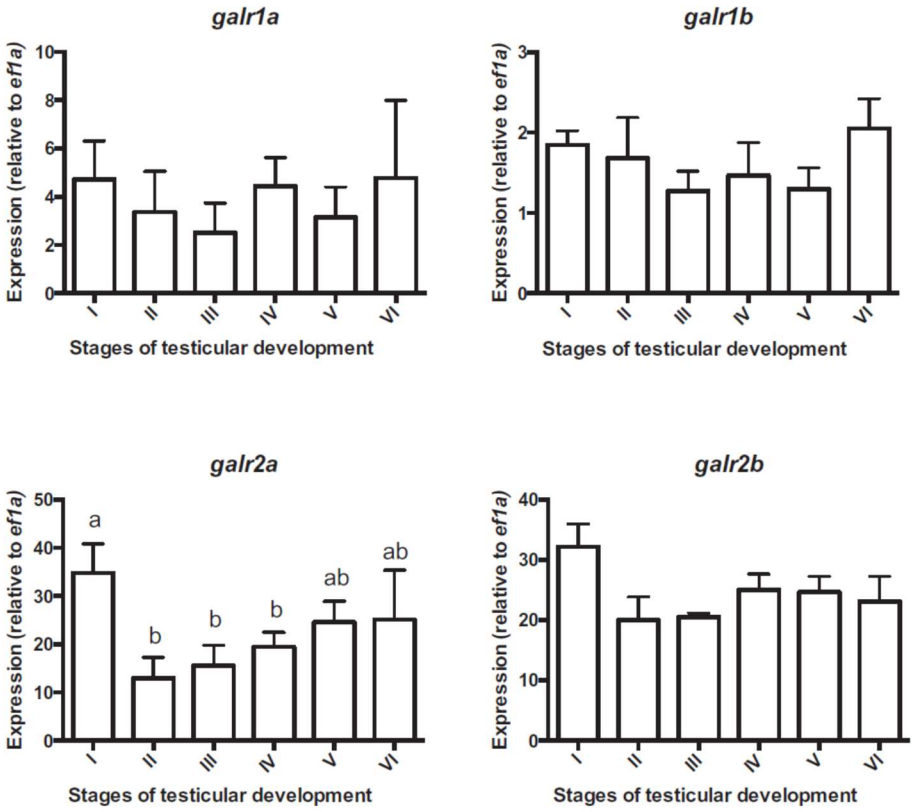

Figure 1. mRNA expression profiles of *galr* transcripts in the hypothalamus of 2-years old male throughout the reproductive season. Expression levels (copy number of target gene normalized by copy number of the reference gene *ef1a*) are expressed as mean ± SEM (n = 3-6), with different lowercase letters denoting statistical differences between testicular development stages.

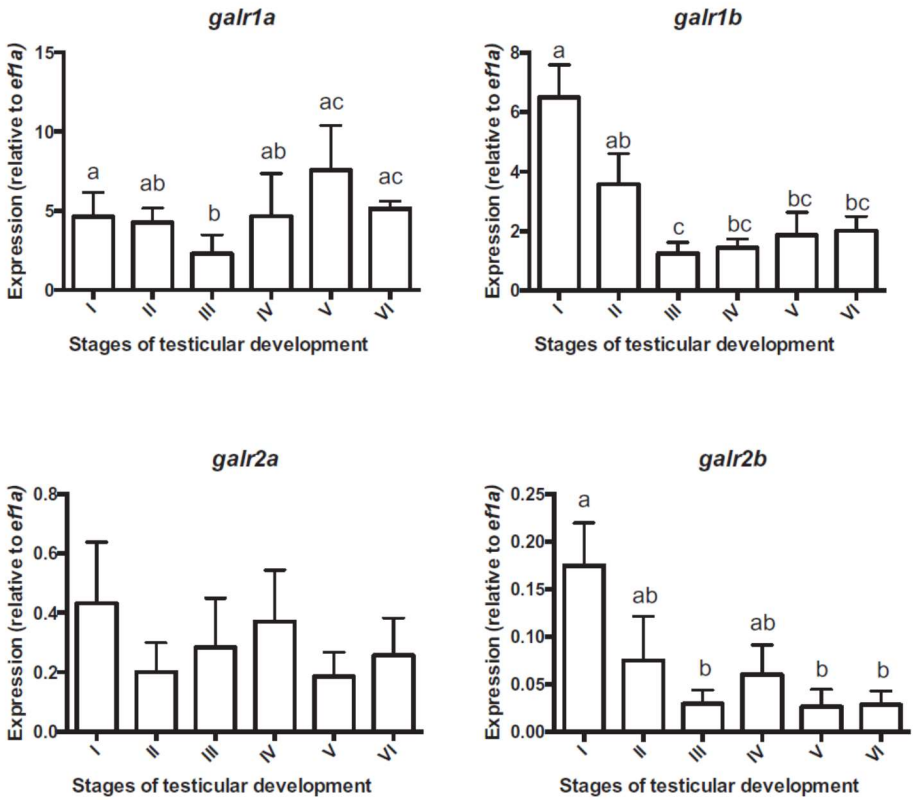

Figure 2. mRNA expression profiles of *galr* transcripts in the pituitary of 2-years old male throughout the reproductive season. Expression levels (copy number of target gene normalized by copy number of the reference gene *ef1a*) are expressed as mean ± SEM (n = 2-6), with different lowercase letters denoting statistical differences between testicular development stages.

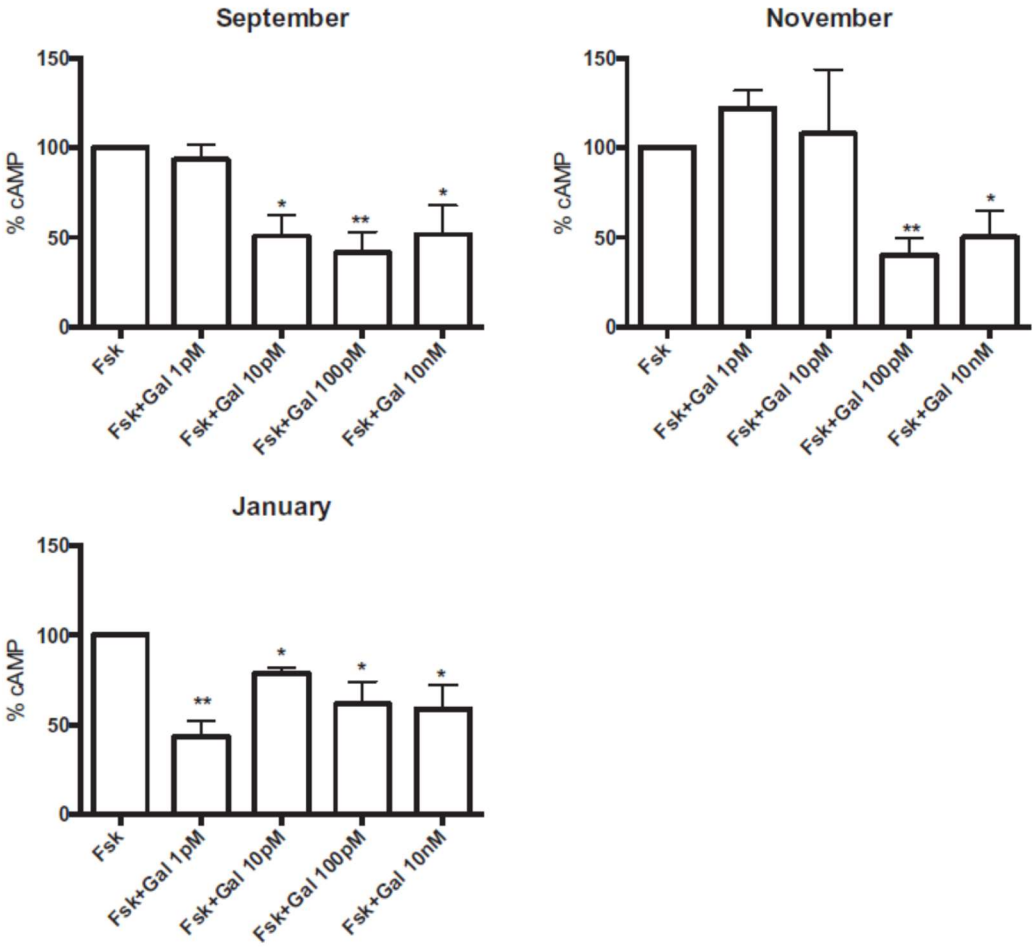

Figure 3. *In vitro* effects of galanin on forskolin-stimulated cAMP production by dispersed pituitary cells from 2-year-old male sea bass at different times of the reproductive cycle. cAMP levels for the different treatments (rat/mouse 1-29 Gal at different concentrations) are expressed as the percentage of cAMP production relative to cells incubated with 200nM forskolin (Fsk). Each value is the mean ± SEM for six independent experiments (prepared from n=5 fish each) and triplicate measurements. * and ** denote significant differences ($P < 0.05$ and $P < 0.01$, respectively) for each treatment compared to the group stimulated with Fsk alone, defined as the control.

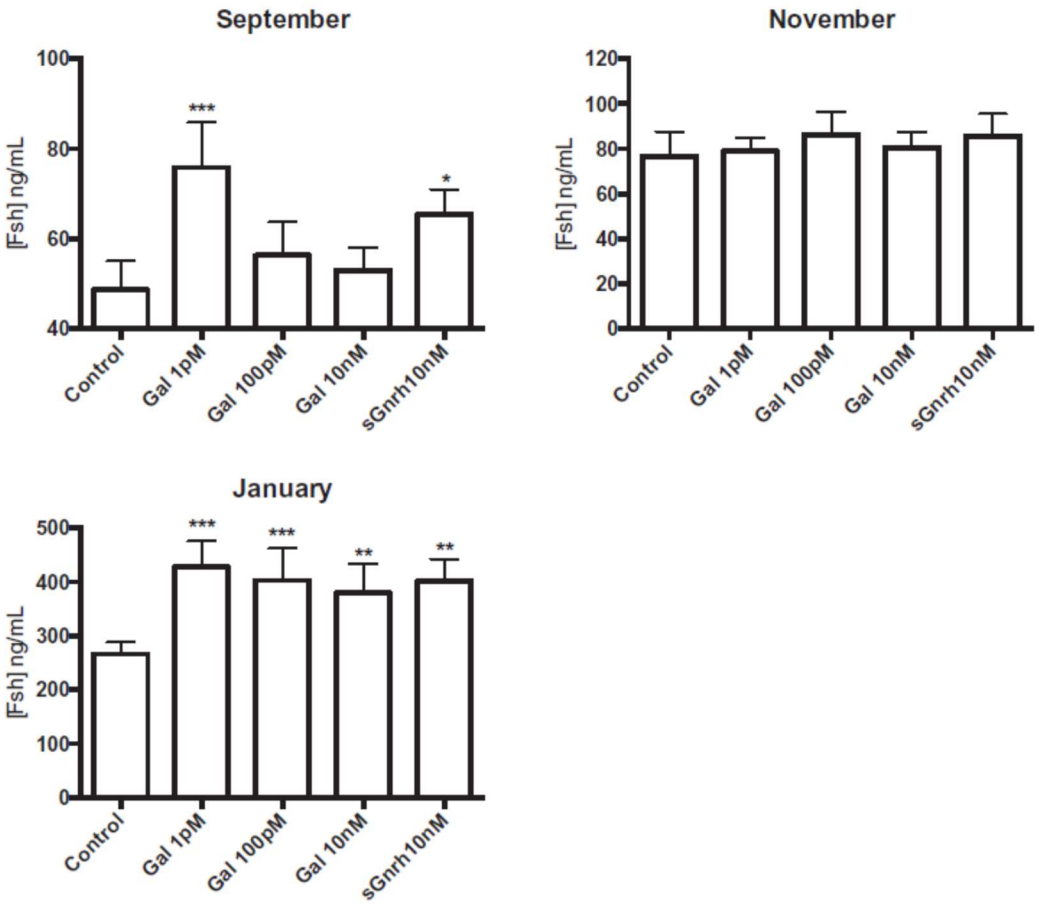

Figure 4. *In vitro* effects of galanin on the release of gonadotropin Fsh by dispersed pituitary cells from 2-year-old male sea bass at different times of the reproductive cycle. Fsh levels presented for the different treatments (rat/mouse 1-29 Gal at different concentrations and sGnrh at 10 nM) are the mean ± SEM for six independent experiments (prepared from n=5 fish each) and triplicate measurements. ***, ** and * indicate significant differences compared to the control ($P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively).

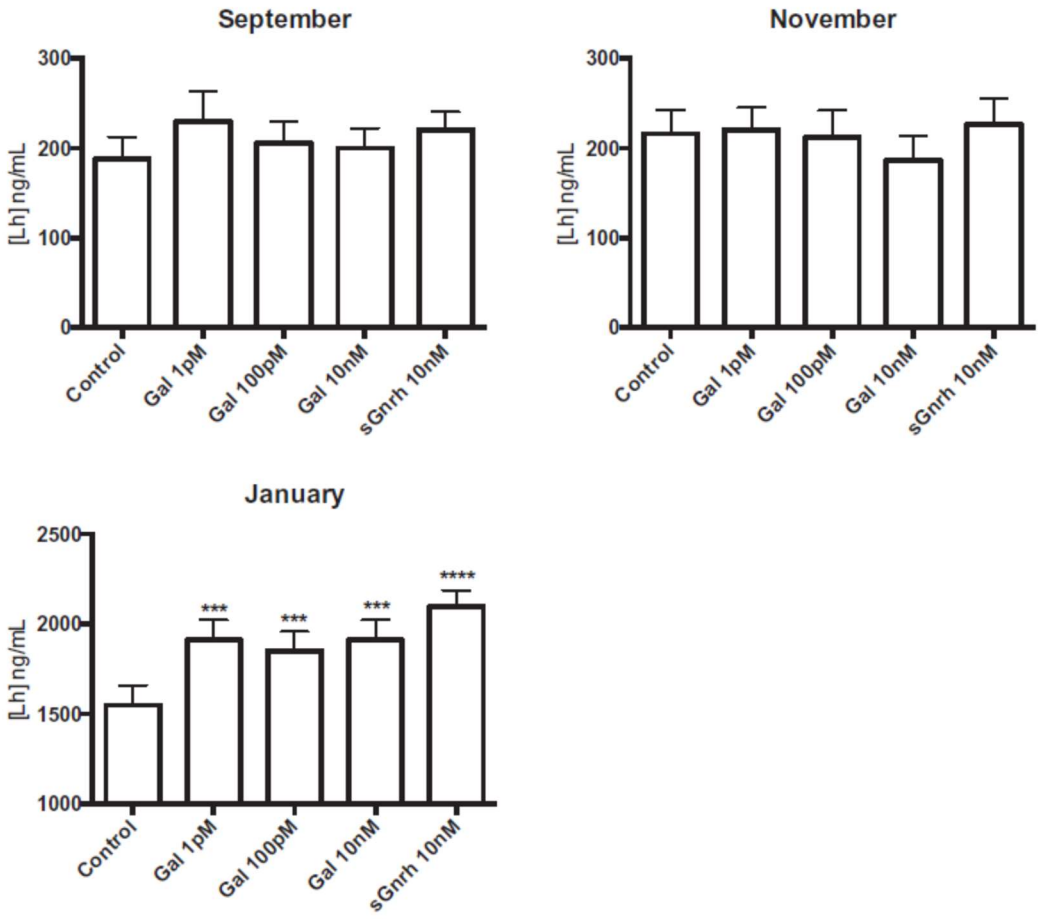

Figure 5. *In vitro* effects of galanin on the release of gonadotropin Fsh by dispersed pituitary cells from 2-year-old male sea bass at different times of the reproductive cycle. Fsh levels presented for the different treatments (rat/mouse 1-29 Gal at different concentrations and sGnrh at 10 nM) are the mean ± SEM for six independent experiments (prepared from n=5 fish each) and triplicate measurements. *** and **** indicate significant differences compared to the control ($P < 0.001$ and $P < 0.0001$, respectively).