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Research paper

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

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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.

1. Introduction

Galanin (Gal)[2] 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 signaling 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).

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.

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[2] Protein and gene nomenclature followed that recommended by genenames.org and used for fish at http://zfin.org/; in abbreviation list, for each case protein abbreviation is presented first followed by the corresponding gene abbreviation.

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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, 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 h light-dark, LD, in winter, and 15:9 in summer) and natural temperature (between 11 °C in winter and 25 °C 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 × 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. (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 −80 °C 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 20 μL 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 (1× 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).