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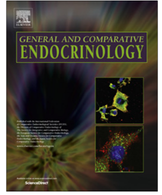

# Novel galanin receptors in teleost fish: Identification, expression and regulation by sex steroids

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ABSTRACT

In fish, the onset of puberty, the transition from juvenile to sexually reproductive adult animals, is triggered by the activation of pituitary gonadotropin secretion and its timing is influenced by external and internal factors that include the growth/adiposity status of the animal. Kisspeptins have been implicated in the activation of puberty but peripheral signals coming from the immature gonad or associated to the metabolic/nutritional status are also thought to be involved. Therefore we hypothesize the importance of the galinergic system in the brain and testis of pre-pubertal male sea bass as a candidate to translate the signals leading to activation of testicular maturation. Here, the transcripts for four galanin receptors (GALR), named *GALR1a*, *1b*, *2a* and *2b*, were isolated from European sea bass, *Dicentrarchus labrax*. Phylogenetic analysis confirmed the previously reported duplication of *GALR1* in teleost fish, and unravelled the duplication of *GALR2* in teleost fish and in some tetrapod species. Comparison with human showed that the key amino acids involved in ligand binding are present in the corresponding GALR1 and GALR2 orthologs. Transcripts for all four receptors are expressed in brain and testes of adult fish with *GALR1a* and *GALR1b* abundant in testes and hardly detected in ovaries. In order to investigate whether *GALR1* dimorphic expression was dependent on steroid context we evaluated the effect of 11-ketotestosterone and 17β-estradiol treatments on the receptor expression in brain and testes of pre-pubertal males. Interestingly, steroid treatments had no effect on the expression of *GALRs* in the brain while in the testes, *GALR1a* and *GALR1b* were significantly up regulated by 11KT. Altogether, these results support a role for the galaninergic system, in particular the *GALR1* paralog, in fish reproductive function.

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## 1. Introduction

Puberty is the developmental period during which an immature juvenile acquires the capacity to reproduce for the first time. The activation of the hypothalamus–pituitary–gonadal (HPG) axis is a key event at the onset of puberty: gonadotropin-releasing hormone (GnRH) secreted by hypothalamic neurons stimulate the pituitary secretion of gonadotropins, that stimulate the production of sex steroids and support gametogenesis in the gonads (Colledge, 2004; Taranger et al., 2010). However, before the full activation of the pulsatile GnRH secretion a series of neuroendocrine events need to occur, which appears to integrate genetic, internal and environmental signals that influence the timing of puberty (Taranger et al., 2010). Kisspeptins and their receptor GPR54 appear to be important in integrating these signals in the brain at puberty onset (Colledge, 2004; Felip et al., 2009), but additional triggers of pubertal GnRH activation are also thought to be involved.

A likely candidate integrator of different signals to trigger puberty onset is galanin (GAL). GAL is a neuropeptide that mediates multiple physiological processes in vertebrates, including learning, nociception, food intake and reproduction (Lang et al., 2007). GAL is mainly localized in the brain and pituitary gland and GAL

*Abbreviations:* 11KT, 11-ketotestosterone; aa, amino acids; dl, *Dicentrarchus labrax*; $E_2$, 17β-estradiol; GAL, galanin; GALR, galanin receptor; GnRH, gonadotropin-releasing hormone; GPCR, G-protein-coupled receptor; HPG, Hypothalamus–pituitary–gonad; ir, immunoreactive; LH, luteinizing hormone; ML, maximum likelihood; T, testosterone.

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immunoreactive (GAL-ir) fibers projecting from the hypothalamic region onto the pituitary are well characterized in fish (Cornbrooks and Parsons, 1991a,b; Moons et al., 1991; Olivereau and Olivereau, 1991; Anglade et al., 1994; Power et al., 1996) and mammals (Ch'ng et al., 1985; Arai et al., 1990; Gai et al., 1990). In the pituitary, GAL is mainly detected in the pars distalis, in close contact with somatotropes, and has been shown to affect growth hormone and prolactin secretion (Bauer et al., 1986; Diez et al., 1992; Wynick et al., 1998).

Interestingly, GAL has also been shown to be co-expressed and/or secreted in luteinizing hormone releasing hormone (LHRH) (Merchenthaler et al., 1991; Marks et al., 1994) and kisspeptin (KISS) neurons (Porteous et al., 2011). Furthermore, GAL was shown to stimulate luteinizing hormone (LH) secretion in the pituitary (Sahu et al., 1994), suggesting a role in the activation of the LH surge and in the activation of gonadal steroid production at puberty onset. Conversely, 17β-estradiol ($E_2$) treatments increased GAL binding in the uterus of immature castrated quails (Tsutsui et al., 1998) and changed the number and/or affinity of galanin-binding sites in several brain regions in rats (Planas et al., 1995), suggesting galanin receptors (GALR) may be regulated by gonadal steroids. The identification of three galanin receptor subtypes (GALR1, GALR2 and GALR3) in human and rats (Habert-Ortoli et al., 1994; Parker et al., 1995; Fathi et al., 1997; Wang et al., 1997a,b; Kolakowski et al., 1998; Pang et al., 1998), allowed confirmation of a direct effect of sex steroids in the regulation of *GALR1* and *GALR2* gene transcription in the brain (Bouret et al., 2000). In addition, changes in *GALR1* gene expression in the preoptic area during the estrous cycle were also detected (Faure-Virelizier et al., 1998), further confirming a regulation of GALRs by sex steroids, as found for the ligand.

In fish, most studies have focused on the role of galanin on feeding regulation (reviewed by Mensah et al. (2010)) and few reports have dealt with its involvement in reproductive functions. As with mammalian species, fish display brain GAL-ir sexual dimorphism (Cornbrooks and Parsons, 1991a,b; Prasada Rao et al., 1996; Jadhao and Meyer, 2000) and in eels, GAL levels vary according to reproductive and physiological stages (Olivereau and Olivereau, 1991). In addition, in a preliminary *in vitro* study with goldfish pituitary cells galanin stimulated LH release (Prasada Rao et al., 1996), suggesting parallelism between fish and mammals in putative GAL reproductive functions. Information on galanin receptors in fish is even scarcer as to date there is only one *in silico* genome survey identifying putative ortholog *GALR* gene duplicates (*GALR1a*, *GALR1b* and *GALR2*) (Liu et al., 2010) and a report correlating the expression of zebrafish (*Danio rerio*) *GALR1a* in the intestine with different feeding regimes (Li et al., 2013).

The European sea bass (*Dicentrarchus labrax*), henceforth designated sea bass, is an aquaculture species with a high incidence of precocious puberty in males, a problem that negatively affects productivity (Taranger et al., 2010). The present study aimed at identifying and characterizing sea bass ortholog GALRs and to investigate whether sex steroid treatments modify their expression in brain and testes of pre-pubertal fish.

## 2. Methods

### 2.1. Animals

Fish (larvae, immature males and adults) were obtained from local fish farms and maintained at the University of Algarve Ramalhete Marine Station (Faro, Portugal) in 500 L through-flow seawater tanks at 17 ± 2 °C and natural photoperiod. All animal maintenance and manipulation procedures were performed in strict compliance with national legislation for the use of laboratory animals under a group-1 license issued by the Directorate-General for Veterinary, Ministry of Agriculture, Rural Development and Fisheries of Portugal.

### 2.2. Steroid treatments

Two independent sex steroid experiments, 11-ketotestosterone (11KT) and 17β-estradiol ($E_2$), were performed with sexually immature male sea bass (209.2 ± 4.4 g) before the beginning of the reproductive season (in November). The treatments consisted of single intra peritoneal injections of coconut oil implants without (control) or with two doses of steroid (0.5 and 5 mg/kg) under anaesthesia (1:10,000 in 2-phenoxyethanol). For each experiment, fish were randomly distributed between 6 tanks ($n = 9$ per tank) – control, two doses of hormone and two sampling times – and left to acclimatize for at least 1 week before treatment. At 12 h and 24 h after treatment one tank from each treatment was euthanized with an overdose of 2-phenoxyethanol (1:5,000), the fish measured (nearest 0.5 cm) and weighted (nearest g) and brain and immature testes dissected and immediately frozen in liquid nitrogen and stored at −80 °C. Blood samples were collected from the caudal vein with heparinized 1 ml syringes (150 U/ml ammonium heparin, Sigma–Aldrich), before treatment and at each sampling point. Plasma was separated by centrifugation and stored at −80 °C until determination of hormone plasma levels.

### 2.3. Radioimmunoassay (RIA) for sex steroids

Sex steroid levels were measured in individual plasma samples by RIA using specific antiserum against 11KT (Kime and Manning, 1982) or against $E_2$ (Guerreiro et al., 2002). All samples from the same experiment were quantified in duplicate in a single assay.

### 2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from frozen tissues using TRI reagent (Sigma–Aldrich) and its integrity and purity was assessed by 1% agarose gel electrophoresis and quantification in a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). Total RNA (8 μg) was treated with DNase (DNA-free kit, Ambion, UK) and cDNA synthesis carried out in 20 μl reactions containing 500 ng of DNase-treated RNA, 200 ng of random hexamers (Jena Biosciences, Germany), 100 U of RevertAid (Fermentas, Thermo Fisher Scientific, USA) reverse transcriptase and 8 U of RiboLockRNase Inhibitor (Fermentas). Reactions were incubated for 10 min at 25 °C and 60 min at 42 °C, followed by enzyme inactivation for 10 min at 70 °C, and storage at −20 °C until use.

### 2.5. Identification and cloning of sea bass GALRs

Mammalian GALR amino acid sequences were used for the initial mining searches against the sea bass genome (Kuhl et al., 2010) using BLAT (Kent, 2002). Four GALR genes were identified, their predicted genomic and cDNA sequences retrieved and used to design specific primers to amplify and clone their coding regions (Table 1). Each sea bass GALR cDNA was amplified by reverse transcription-polymerase chain reaction (RT-PCR) in 25 μl containing 1 μl of cDNA (from whole larvae, or brain or liver of adult sea bass), 10 pmol each primer (Table 1), 40 μM dNTPs and 0.5 U DreamTaq DNA Polymerase (Fermentas), in 1× DreamTaq buffer. Cycling conditions were 5 min at 95 °C, 35 cycles of 20 s at 95 °C, 20 s at the optimized annealing temperature for each primer pair (Table 1) and 1 min at 72 °C, followed by 5 min at 72 °C. Amplified targets were gel-purified, inserted into pGEM-T Easy (Promega, Southampton, UK) and identity confirmed by sequencing. Positive clones

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