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

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Abbreviations

11KT, 11-ketotestosterone; aa, amino acids; dl, *Dicentrarchus labrax*; E_2, 17β-estradiol ; GAL, galanin; GALR, galanin receptor; GnRH, gonadotrophin-releasing hormone ; GPCR, G-protein-coupled receptor; HPG, hypothalamus-pituitary-gonad; ir, immuno reactive; LH, luteinizing hormone; ML, maximum likelihood; T, testosterone.

Abstract

In fish, the onset of puberty, the transition from juvenile to sexually reproductive adult animals, is triggered by the activation of pituitary gonadotrophin secretion and its timing is influenced by external and internal factors that include the growth/adiposity status of the animal. Kisseptins 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. Additionally, there is evidence that the galaninergic system in the brain and testis of pre-pubertal male sea bass is a possible mediator involved in the translation of somatic signals leading to gonadal 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 orthologues. 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.

Keywords: galanin receptors, puberty, sea bass, estradiol, 11-ketotestosterone

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 or reproduction (Lang et al. 2007). Initially isolated in porcine intestine (Tatemoto et al. 1983), GAL is widely distributed in the central and peripheral nervous systems and in the endocrine system, and multiple studies have highlighted its key role as an important messenger within the nervous system and the neuroendocrine axis (Lang et al. 2007). GAL is mainly localized in the brain and pituitary gland. GAL immunoreactive (GAL-ir) fibers projecting from the hypothalamic region onto the pituitary are well characterized in fish (Cornbrooks and Parsons 1991b; 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; 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; 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 orthologue GALRs 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 orthologue 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 ºC 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 one 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 ºC. Blood samples were collected from the caudal vein with heparinized 1ml syringes (150 U/ml ammonium heparin, Sigma-Aldrich), before treatment and at each sampling point. Plasma was separated by centrifugation and stored at -80 ºC 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 ºC and 60 min at 42 ºC, followed by enzyme inactivation for 10 min at 70 ºC, and storage at -20 ºC until use.

2.5. Identification and cloning of sea bass *GALR*s

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 1x DreamTaq buffer. Cycling conditions were 5 min at 95 ºC, 35 cycles of 20 sec at 95 ºC, 20 sec at the optimized annealing temperature for each primer pair (Table 1) and 1 min at 72 ºC, followed by 5 min at 72 ºC. Amplified targets were gel-purified, inserted into pGEM-T Easy (Promega, Southampton, UK) and identity confirmed by sequencing. Positive clones for the four *GALR* isoforms were re-sequenced to give at least 3-fold coverage across their entire coding regions. Sequence polymorphisms were carefully confirmed and the final sea bass *GALR* cDNA sequences were assembled with the CAP contig assembling program (Huang 1992).

2.6. Sequence characterization and phylogenetic analysis

Genomic organization of sea bass GALR genes was characterized from the alignment of each genomic sequence with the sequence of the respective cDNA. Multiple sequence alignments of deduced amino acid sequences for sea bass GALRs with human GALRs were carried out using ClustalW v.2.0 (Larkin et al. 2007) (see legend of Figure 2 for accession numbers of sequences used) and edited using GeneDoc version 2.7.0 (Nicholas et al. 2007). Transmembrane regions were predicted from the deduced sea bass GALR protein sequences using TMHMM v. 2.0 (Sonnhammer et al. 1998) and phosphorylation and N-glycosylation sites using NetPhos and NetNGlyc (Blom et al. 2004).

The presence of GALRs in different vertebrate species was investigated using sea bass and human amino acid sequences as initial query in tBLASTN against publicly available DNA databases (Ensemble for genomes and Genbank for mRNAs and expressed sequence tags) or BLASTP against

protein databases (Genbank) (Altschul et al. 1997). Phylogenetic analyses were conducted using the maximum likelihood (ML) method (PhyML 3.0 aLRT) (Anisimova and Gascuel 2006) with 100 bootstrap replicates on a JTT substitution model with a discrete gamma distribution of rates among sites with 4 categories, using the retrieved seven transmembrane domain regions of galanin receptors based on a ClustalW 2.0 alignment (Larkin et al. 2007) analysed with PROTTEST (version 2.4), to select the model of protein evolution that best fits the dataset (Abascal et al. 2005). A search for the optimal ML tree was also performed.

2.7. Analysis of GALR gene expression

The distribution of GALR transcripts was analysed by semi-quantitative reverse-transcription (RT-PCR) using isoform-specific primers (Table1) in cDNAs from several tissues of male and female adult sea bass: liver, gonads, olfactory epithelium and brain dissected into three different regions, anterior brain (olfactory bulb and telencephalon), middle brain (optic tectum and diencephalon) and posterior brain (cerebellum, medulla oblongata and part of the spinal cord). Reaction conditions were as described for cloning (section 2.5) and cycling conditions were 5 min at 95 ºC, 35 cycles of 10 sec at 95 ºC, 10 sec at optimized annealing temperatures (Table 1) and 10 sec at 72 ºC, followed by 2 min at 72 ºC. PCR products were analysed in 2% agarose gel electrophoresis and sequenced to confirm identity.

Transcript levels of the four *GALR* genes in immature male sea bass brain and testis in response to sex steroid treatments were measured by real time RT-PCR (qPCR) in a Bio-Rad iClycler iQ5 qPCR thermocycler in six individual fish from each experimental group. Quantifications were carried out in duplicate using the relative standard curve method and the EvaGreen chemistry, in 15 µl reactions containing 2 µl of each cDNA (diluted 1:10), 300 nM each specific primer (Table 1) and 1x Sso Fast EvaGreen Supermix (Bio-Rad Laboratories, USA). Cycling conditions were 30 sec at 95 °C, 45 cycles of 5 sec at 95 ºC and 10 sec at the optimized annealing temperature (Table 1), followed by a final melting curve between 65 and 98 °C. Specificity of qPCR reactions for all genes was confirmed by the presence of single peaks in each melt curve, amplification of expected size bands analysed by 2% agarose gel electrophoresis and sequencing of qPCR products. A cDNA synthesis sample without reverse transcriptase (-RT control for the absence of genomic DNA contamination) was tested for all genes and no amplification was detected. Standard curves relating amplification cycle with initial template quantity were generated using serial dilutions of specific RT-PCR products for each gene (obtained from brain cDNA using the same primers) and included in all quantification plates, with qPCR efficiency ranging between 85–97% with R^2>0.98.

Candidate reference genes tested included the 18S ribosomal RNA sub-unit (18S), beta actin (β-actin) and elongation factor 1α (EF1α). The combinations showing the least variation between cDNA samples and between experimental treatments and thus chosen to normalize expression data were the geometric mean of β-actin and EF1α, in the case of the treatments with 11KT and the geometric mean between 18 S and EF1α for the treatments with E_2. Relative expression levels were calculated by dividing detected copy number between the target and the geometric mean of two reference genes, for each cDNA sample.

2.8. Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Differences between groups were analysed by two-way analysis of variance (SigmaStat, v.3.50, Systat Software, Inc, San Jose, CA, USA) using steroid treatment and sampling time as factors on log2 transformed data of relative expression values or plasma sex steroid levels, followed by the Holm-Sidak test. The level of significance was set at $p< 0.05$.

3- Results

3.1. Identification and characterization of four GALR genes in sea bass

Four distinct *GALR* genes (Figure 1) were identified in the genome of the sea bass (designated by the prefix dl) and their corresponding coding region cDNA isolated. They were named *dlGALR1a*, *dlGALR1b*, *dlGALR2a* and *dlGALR2b* according to their positions in the phylogenetic tree (see section 3.2).

The *dlGALR1a* 1053 bp cDNA (*GenBank:* KF878115) was cloned from whole larvae and encoded a predicted protein of 350 amino acids, while the 1074 bp *dlGALR1b* (*GenBank:* KF878116) was cloned from liver and encoded a protein of 357 amino acids. Both genes have a structural organization of three coding exons. One large exon encodes their NH_2 terminal end and predicted first five transmembrane (TM) domains characteristic of G-protein-coupled receptors (GPCRs)- see section 3.2. A small (66 bp) exon encodes the third intracellular loop and the third exon encodes the remaining of the receptor protein (Figures 1 and 2).

The *dlGALR2b* 1137 bp cDNA (*GenBank:* KF878117) was cloned from sea bass brain and encoded a protein of 378 amino acids. Two *dlGALR2a* cDNA clones of 981 bp were amplified from liver and encoded a 326 amino acids protein but differed in 13 nucleotides (seven amino acids), and may represent possible polymorphisms in this gene (*GenBank* KF878118, clone E1 and KF878119, clone E4). Since the E1 transcript was the one with highest similarity to the genomic sequence (8 of 13

identical nucleotides with 5 of 7 identical amino acid residues in the polymorphic positions), this sequence was the one further used in sequence alignments and phylogenetic analysis. Both *dlGALR2a* and *2b* genes are organized in two exons separated by a large intron of approx. 3.6 kb, dividing the predicted GALR proteins in the second intracellular loop (Figures 1 and 2). Consensus sequences were observed at all intron donor and acceptor splice sites (GT/AG) for the four *dlGALR* genes.

3.2. Sequence comparison and phylogenetic analysis

The deduced amino acid sequences of dlGALRs were compared with human GALRs (hGALR1 to 3). dlGALR1a and dlGALR1b shared, respectively, 73 and 67 % identities with hGALR1 (Figure 2) and only 26-31 % to hGALR2 and hGALR3. dlGALR2a and dlGALR2b shared, respectively, 54 and 50 % identities with hGALR2, 44 % to hGALR3 and 32-33 % to hGALR1.

Hydropathy analysis of dlGALRs predicts the typical structure of members of the GPCR superfamily: an extracellular N-terminus, a cytoplasmic C-terminus and seven transmembrane spanning regions (TM). Other characteristic features found in GPCRs were also found in all dlGALR deduced sequences, including the two cysteine residues in the first and second extracellular loops that may form a disulphide bond, consensus sites for N-linked glycosylation in the N-terminal region and the aromatic triplet signature (DRY) adjacent to TM3 (Figure 2).

Comparison of the deduced amino acid sequence for the dlGALR1 duplicates shows a remarkable conservation to mammalian GALR1. The most variable regions are the N- and C-termini and the second extracellular loop. However, closer inspection of the physico-chemical properties of the amino acids in the N- and C- termini shows also striking conservation between the C-terminus of fish and mammalian GALR1 proteins (Supplementary file 1). In addition, dlGALR1 duplicates contain the essential characteristic features of mammalian GALR1 proteins (Figure 2), including three serine residues for potential protein kinase C phosphorylation, two potential cAMP/cGMP protein kinase phosphorylation sites, and C-terminal cysteine residues that can serve as potential palmitoylation sites. The key amino acids for galanin binding identified in hGALR1 (His264, Phe282 and Glu271) are also conserved.

Comparison of amino acid sequences for the GALR2 forms shows that the physico-chemical properties of the amino acids of both N- and C- termini are very well conserved between dlGALR2b and hGALR2 (Supplementary figure 1). It should be noted, however, that the dlGALR2a has shorter N- and C- termini than dlGALR2b and hGALR2. Nevertheless, both duplicate dlGALR2 show high conservation of the key amino acids identified for galanin binding in hGALR2 (His252, His253,

Phe264 and Tyr271), although dlGALR2b has the substitution of His252 by a tyrosine residue (Figure 2).

The search for protein sequences and *GALR1* genes in other vertebrate genomes allowed the identification of similar gene duplications. Interestingly, *GALR2* duplicate genes were also identified in other teleosts, e.g. in zebrafish (*Danio rerio*) and cichlids, as well as in tetrapod species, e.g. in *Xenopus* and chicken (*Gallus gallus*), establishing that this gene duplication is not specific to the teleost lineage. Phylogenetic analysis (Figure 3) of all identified *GALR*s from fish and tetrapods have resulted in the separation of two main clades, a *GALR1* and a *GALR2*/*GALR3* clade. No *GALR3* orthologue could be identified in teleosts, although, contrary to previous believed, we have established that this receptor is not exclusive of placental mammals but could be found in Amphibia (*Xenopus* and green anole, *Anolis carolinensis*), Aves (chicken, *Gallus gallus*) and Metatheria (opossum, *Monodelphis domestica*) (Figure 3).

3.3. Tissue distribution of sea bass GALRs and regulation by steroid hormones

Transcripts for the four *dlGALR*s were detected in different regions of the brain of adult male and female sea bass (Figure 4). In addition, the four *dlGALR*s were also expressed in peripheral tissues, such as gonads and olfactory epithelium. In particular *dlGALR1a* and *GALR1b* appeared to be more abundant in testis compared to ovary.

Upon exogenous administration of E_2 and 11KT to pre-pubertal male sea bass, blood plasma levels of each of these steroids increased for both concentrations at 12 or 24 h post injection (Figure 5). In the brain, no significant changes were observed for *GALR* transcripts in response to any of the steroid treatments (Figure 6 and 7). In the testis, *GALR1a* at the lowest 11KT concentration and *GALR1b* at both 11KT concentrations were significantly up regulated compared to control at 24 h (Figure 6).

4- Discussion

The present study identified two paralog pairs of GALR genes in sea bass, confirming a teleost specific duplication of *GALR1* genes (*dlGALR1a* and *dlGALR1b*) and identifying novel *GALR2* gene duplications in sea bass and tetrapod species, indicating a duplication prior to the teleost lineage. Additional characterization of these receptors suggested both *dlGALR1* genes to be sensitive to 11KT in the testes of pre-pubertal fish.

The genomic structure and deduced amino acid sequences of dlGALRs are highly conserved with the human GALR1 and GALR2 receptors. The sea bass *GALR1* duplicate genes have a conserved genomic structure composed by three coding exons as previously described for human and mouse *GALR1* genes (Jacoby et al. 1997; Iismaa et al. 1998). The first exon spans from the putative N-terminal onto the end of transmembrane region 5, the second exon includes the third intracellular loop and the third exon spans from transmembrane region 6 onto the putative C-terminus. The sea bass *GALR2* gene duplicates also have a conserved two exon gene structure when compared to the mammalian orthologue *GALR2* (Iismaa et al. 1998; Pang et al. 1998). The *dlGALR2* duplicates first exon spans from the putative N-terminal onto the end of transmembrane region 3 and the second exon encodes from the second intracellular domain until the putative C-terminus.

Comparison of dlGALRs deduced amino acid sequences with that of human GALRs demonstrate that the GALR1 orthologue sequences have diverged less than those of GALR2. Since often duplicate genes undergo divergent evolution through sub-functionalization, loss or gain of new functions (Prince and Pickett 2002), it is surprising that both gene products of the two fish GALR1 paralogues have maintained the key features that characterize the mammalian GALR1, including the key amino acids for its selective binding to the galanin peptide (Kask et al. 1998; Church et al. 2002). In contrast, the *dlGALR2* duplicate sequences not only have less conservation with their mammalian orthologue as well as differ in several key features. Indeed, mutagenesis and analysis of three-dimensional models for the hGALR2 have identified amino acids that are responsible for its ligand binding activity and have underlined the importance of the N-terminus in this process (Lundström et al. 2007; Jurkowski et al. 2013). In this respect, dlGALR2b is the most structurally similar to hGALR2, as it has highly conserved N- and C-termini and conservation of all the key amino acids for ligand binding. In contrast, dlGALR2a has a shorter N- terminus, more similar to what is seen in hGALR3 and a shorter C-terminus like human and sea bass GALR1 receptors. It also shows polymorphisms although they correspond to conserved substitutions and apparently not in position encoding amino acids identified as important. These differences suggest dlGALR2a may have acquired different functions from those of dlGALR2b in sea bass. Nevertheless, ligand

activation and functional studies will be necessary to determine the specificities between these novel receptors in sea bass.

A recent report identified *GALR1* and *GALR2* genes from fish to mammals (Liu et al. 2010). Interestingly, our search for human GALR2 orthologues allowed the identification of previously unsuspected duplicated *GALR2* in sea bass. Indeed, this novel gene duplication is present not only in teleost fish (e.g. sea bass, zebrafish and cichlid species) but also in some tetrapods (e.g. *Xenopus* and chicken). In addition, we have searched for *GALR3* orthologues but failed to find it in fish genomes. However, unlike previous assumptions (Liu et al. 2010), the *GALR3* gene is not exclusive of eutherian mammals and it could be confirmed also in amphibian, bird and marsupial genomes. Thus, phylogenetic analysis of teleost and tetrapod *GALRs* resulted in the separation of two major clades formed by *GALR1* and *GALR2/GALR3* genes, suggesting that the two clades emerged from a common ancestral receptor and underwent distinct evolutionary trajectories, the teleost fish having lost *GALR3* and mammals one of the *GALR2* duplicates.

Gene expression analysis suggest the brain as the main site of *GALR*s transcription in agreement to what has been described for mammals (reviewed in Mensah et al. 2010; Webling et al. 2012). Interestingly, we have detected a sexual dimorphism in the expression of *dlGALR1a* and *dlGALR1b* in adult ovary and testis, as these receptors are expressed in testes but have very low expression in the ovaries, as previously reported for rat *GALR1* (Sullivan et al. 1997). These results are different from what has been reported for zebrafish where only one duplicate *GALR1* gene was expressed in the ovaries and testis (*zfGALR1b*) and its expression was not dimorphic (Li et al. 2013). Nevertheless, it should be stressed that there is great variability in tissue distribution of the different *GALR* subtypes in vertebrates and significant differences in *GALR1* expression in peripheral tissues are also found between human and mice (Wang et al. 1997b). Furthermore, future studies characterizing the tissue distribution and localization of *GALR* genes in more fish species will contribute to elucidate if differential patterns are detected for duplicate genes, or if expression is developmental stage dependent. To date, the only gene expression data on fish *GALR* genes are in zebrafish *GALR1a/1b* (Li et al. 2013), the data from the present study on the four *dlGALRs* and studies reporting the presence of galanin binding sites (suggestive of receptor expression) in the brain and pituitary of some fish species, including sea bass (reviewed by Mensah et al. 2010).

The sexual dimorphism in *dlGALR1a* and *dlGALR1b* receptors found in the gonads of adult fish led us to hypothesize that the gene expression difference between female and males might be attributed to the differences in steroid production between female and male gonads. Interestingly we have found that the expression of *dlGALRs* in the brain was not affected by sex steroids. In rats, GAL is involved in the regulation of androgen production in males at various levels of the HPG axis. In the

brain, GAL amplifies the stimulatory actions of GnRH on LH secretion to sustain the activation of androgen production after pubertal onset (Merchenthaler et al. 1991; Miller et al. 1993; Marks et al. 1994; Scheffen et al. 2003). In addition, it is also involved in potentiating the human chorionic gonadotropin-induced testosterone (T) release in adult testes (Romanelli et al. 1998; Pandit and Saxena 2007), although this effect is not detected in immature individuals (Pandit and Saxena 2007). In the pituitary of male rats GAL is able to stimulate LH secretion but only in the presence of T (Scheffen et al. 2003). The GAL dependence on androgen levels was further corroborated by experiments showing that GAL levels and GAL induced LH secretion was abolished by castration of male rats but could be restored by T replacement (Miller et al. 1993; Scheffen et al. 2003). Interestingly, the few studies focusing on the effect of steroids in GAL receptors have also corroborated this steroid dependency, as in rats *GALR1* and *GALR2* expression in proopiomelanocortin POMC neurons is also abolished by castration and fully restored by T administration (Bouret et al. 2000). Collectively these studies show that GAL is important for the regulation of steroid production, but apparently only after sex steroid production has been activated. These observations support the idea that GAL is not involved in triggering the onset of pubertal development but rather, in amplifying and sustaining the pulsatile activation of LH production throughout this process. Thus, taking this into account, it is possible that the lack of responsiveness of *dlGALRs* in the brain of male pre-pubertal sea bass may reflect the immaturity of the HPG axis at this stage in the individuals analysed and lack of the appropriate steroid context to detect effects on *GALR* gene expression. However, it may also indicate that regulation may occur post-transcriptionally and/or at the level of the ligand.

Nevertheless, we did find a significant stimulatory effect of 11KT on *dlGALR1a* and *dlGALR1b* gene transcription in the testis. In sea bass males, the levels of T and 11KT in the plasma are very low during the first year of development (Rodrıguez et al. 2000) and at the onset of gametogenesis the levels of 11KT are elevated to support the initiation of spermiogenesis and testicular maturation (Rodrıguez et al. 2004). In the present study, the levels of 11KT in the plasma of control fish are within the reported values for pre-pubertal male sea bass (Rodrıguez et al. 2000; Rodríguez et al. 2001) and the administration of 0.5 mg/kg 11KT elicited an increase in plasma steroid levels comparable to those of males going through first spawning (Rodrıguez et al. 2000). Thus, it is interesting that at physiological levels, 11KT was able to stimulate the transcription of both *dlGALR1a* and *dlGALR1b* in the immature testis. The significance of these findings will require further investigation, although it is possible that the sea bass galaninergic system may have a role in the testicular germ cell differentiation. Indeed, upregulation of *GAL* expression is correlated to the pluripotency of human embryonic stem cells (ESC), as described for the ESC markers *NANOG* and

SOX2 (Assou et al. 2007), and in mouse Leydig cells, *GALR1* expression is inversely correlated to the stage of differentiation of these testicular cells (Ohta et al. 2002).

The human *GAL* gene promoter contains conserved estrogen responsive elements (EREs) half sites that are able to be regulated by E_2 (Kofler et al. 1995; Howard et al. 1997) and there are several studies focusing on the effect of E_2 in the expression and/or secretion of GAL in either the brain or pituitary of males (Kaplan et al. 1988; Selvais et al. 1995). Although, in the present study, we did not find significant effects of E_2 on *dlGALRs* expression in the brain or in the testes at 12h or 24h post injection, we cannot discard the possibility for rapid and transient effects occurring between 0 and 12 hours of treatment. Nevertheless, future studies will help to clarify a possible role of E_2 in the regulation of GALRs in sea bass.

In summary, orthologous gene duplicates of vertebrate *GALR1* and *GALR2* were isolated in sea bass and phylogenetic analysis revealed previously unreported *GALR2* orthologues in teleost fish and tetrapods. All dlGALRs are expressed in male and female brain and the expression of *dlGALR1a* and *dlGALR1b* appears to be sexually dimorphic, with higher expression in testis than in ovary. Finally, the expression of both receptors was up regulated by 11KT in pre-pubertal sea bass testis. These results suggest that the galaninergic system, in particular the GALR1 isoforms may play a role in sea bass male reproductive function.

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Figure captions

Figure 1. Genomic organization of sea bass *GALRs* and comparison with the predicted GALR protein structure. In *GALR* genes, exons are represented by white blocks and introns by lines. In GALR proteins, the predicted seven transmembrane domains characteristic of GPCRs are indicated by blocks and numbered. Only the coding regions were analysed and exons were numbered according to mammalian GALR genes (Jacoby et al. 1997; Iismaa et al. 1998). The figure is drawn to scale (scale bar indicates 500 base pairs, bp) and the region spanned by each gene is indicated in base pairs below each gene name.

Figure 2. Multiple sequence alignment of the sea bass deduced GALR proteins with human (h) GALR proteins (Accession numbers hGALR1- NP001471.2; hGALR2- NP003848.1; hGALR3- NP003605.1). Amino acid conservation is shaded and transmembrane domains (TM1-7) predicted by hydropathy analysis and from comparison to mammalian GALRs, are boxed. Conserved residues represented include: potential sites for N-linked glycosylation in the N-terminal region (underlined), two cysteine residues that may for a disulfide bond (black squares), three serine residues for potential protein kinase C phosphorylation (SRR/SKK/SKK in hGALR1, indicated by *), two potential cAMP/cGMP protein kinase phosphorylation sites (SSS/KKK, indicated by black stars), C-terminal cysteine residues for potential palmitoylation (#), the aromatic triplet signature of GPCRs adjacent to TM3 (DRY, indicated by $) and amino acids identified as important for galanin binding to hGALR1 (black arrows) or to human GALR2 (white arrows). The position of the two introns in the *dlGALR1a* and *1b* genes is indicated by black triangles while the intron of *dlGALR2a* and *2b* genes is indicated by a white triangle.

Figure 3. Phylogenetic tree with fish and tetrapod GALR subtypes. Accession numbers are indicated for all sequences and sea bass GALRs are underlined. Consensus phylogenetic tree (maximum likelihood method, 100 bootstrap) produced with the transmembrane domains of GALRs. The *Caenorhabditis elegans* NPR9 was used to root the tree. The bootstrap values for each fork are indicated.

Figure 4. RT-PCR of sea bass galanin receptors in tissues from two males and two females. Bp (posterior brain), Bm (middle brain), Ba (anterior brain), Ov (ovary), Olf (olfactory epithelium), Lv (liver) and T (testis).

Figure 5. Circulating plasma levels of 17β-estradiol (E_2) (A) or 11KT (B) in immature male sea bass immediately before (0 h), 12 and 24 h after implantation with coconut oil containing 0.5 or 5 mg/kg body weight of the respective hormone, or coconut oil alone (control). Each value is the mean ± SEM of 9 fish per group at each sampling time. Asterisk (*) indicates significant differences from the control at each sampling point ($P<0.05$).

Figure 6. Transcript levels of *dlGALRs* in the brain and testis of immature sea bass in response to 11-ketotestosterone (11KT) treatment, measured by qPCR. Each value is the mean ± SEM (n= 6 fish per group at each sampling time) of the relative expression levels 12 and 24 h after implantation with coconut oil containing 0.5 or 5 mg/kg body weight of 11KT or coconut oil alone (control). Asterisk (*) indicates significant differences from the control at each sampling point ($P<0.05$).

Figure 7. Transcript levels of *dlGALRs* in the brain and testis of immature sea bass in response to 17β-estradiol (E_2) treatment, measured by qPCR. Each value is the mean ± SEM (n= 6 fish per group at each sampling time) of the relative expression levels 12 or 24 h after implantation with coconut oil containing 0.5 or 5 mg/kg body weight of E_2 or coconut oil alone (control). There were no statistically significant differences ($p<0.05$) from control for any of the receptor genes and treatments.

Supplementary figures

Supplementary figure 1- Alignment of the N- or C-terminus of the sea bass (*Dicentrarchus labrax*, dl) deduced GALR proteins with human (h) GALR proteins (*Genbank* accession numbers hGALR1- NP001471.2; hGALR2- NP003848.1; hGALR3- NP003605.1). The amino acid physico-chemical properties are shaded (PhysioChemical Prop shading in GeneDoc v 2.7.0, (Nicholas et al. 2007)) and transmembrane domains (TM) predicted by hydropathy analysis are indicated by overlining.

Tables

Table 1- List of primers used in this study, to clone the full-length coding region of each sea bass GALR and for gene expression analysis by semi-quantitative (tissue distribution) and quantitative RT-PCR (expression in response to sex steroid treatments).

Objective	Gene name	Fw/Rv [a]	Primer sequence (5'-3')	Ta [b]	bp [c]
Cloning	*GALR1a*	Fw	ATGAACTTATCAGAGCCCG	64	1053
		Rv	TCAAACGTTGGTGCAGTTAGTCG		
	GALR1b	Fw	ATGGAGCTACAGGTGGAG	56	1074
		Rv	TCAAAGCATTTTACTGGTCTGAG		
	GALR2a	Fw	ATGAACCTCTCCTCGGCGAAC	64	981
		Rv	TCAGTTGGACGTCTCCACGCTGCTC		
	GALR2b	Fw	ATGTCTGACTTTGAGGATTTCAGC	62	1137
		Rv	TCAAGTCTGCCGCTGAAAG		
RT-PCR	*GALR1a*	Fw	GCTGCCCACTGCCTGGCG	62	194
		Rv	CAAACGTTGGTGCAGTTAGTCG		
	GALR1b	Fw	CTGGTGCCGGTTGCCCAGC	60	132
		Rv	CAAAGCATTTTACTGGTCTGAG		
	GALR2a	Fw	GCGTACACCAACTCCTGC	60	165
		Rv	GTTGGACGTCTCCACGCTGCTC		
	GALR2b	Fw	CCATGTGGTTCATGTAGCC	60	169
		Rv	CTGCCGCTGAAAGGGTAAATTC		
	18S	Fw	TGACGGAAGGGCACCACCAG	60	158
		Rv	AATCGCTCCACCAACTAAGAACGG		
	β-Actin	Fw	TGACCTCACAGACTACCT	58	176
		Rv	GCTCGTAACTCTTCTCCA		
	EF1α	Fw	GACACAGAGACTTCATCAAG	58	114
		Rv	GTCCGTTCTTAGAGATACCA		

[a] Forward (Fw) or reverse (Rv) primers; [b] Optimized annealing temperature used for each pair of primers. [c] Amplicon size in base pairs (bp).

Figure1

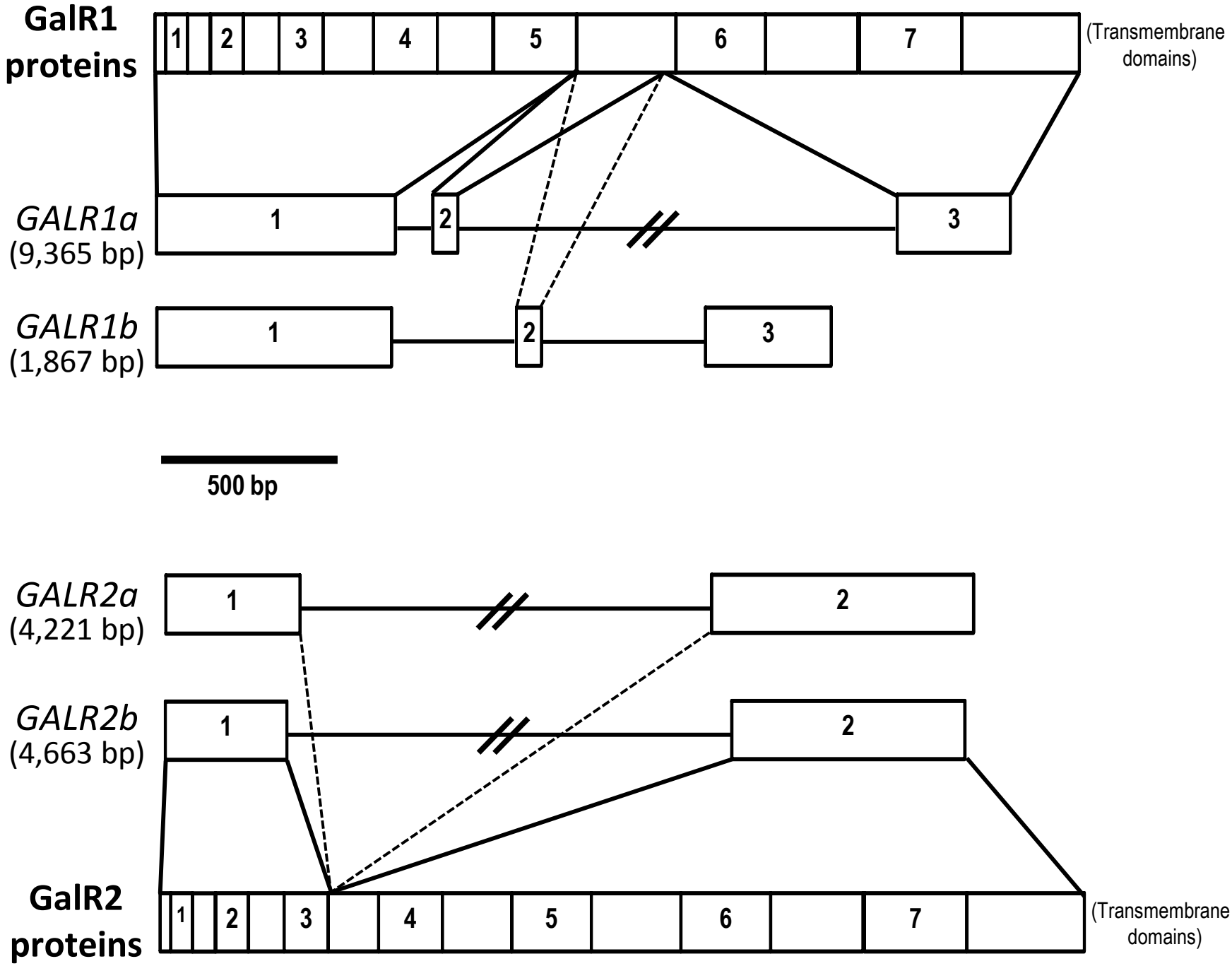

GalR1 proteins
1
2
3
4
5
6
7
(Transmembrane domains)
GALR1a (9,365 bp)
1
2
3
GALR1b (1,867 bp)
1
2
3
500 bp
GALR2a (4,221 bp)
1
2
GALR2b (4,663 bp)
1
2
GalR2 proteins
1
2
3
4
5
6
7
(Transmembrane domains)

Figure 2

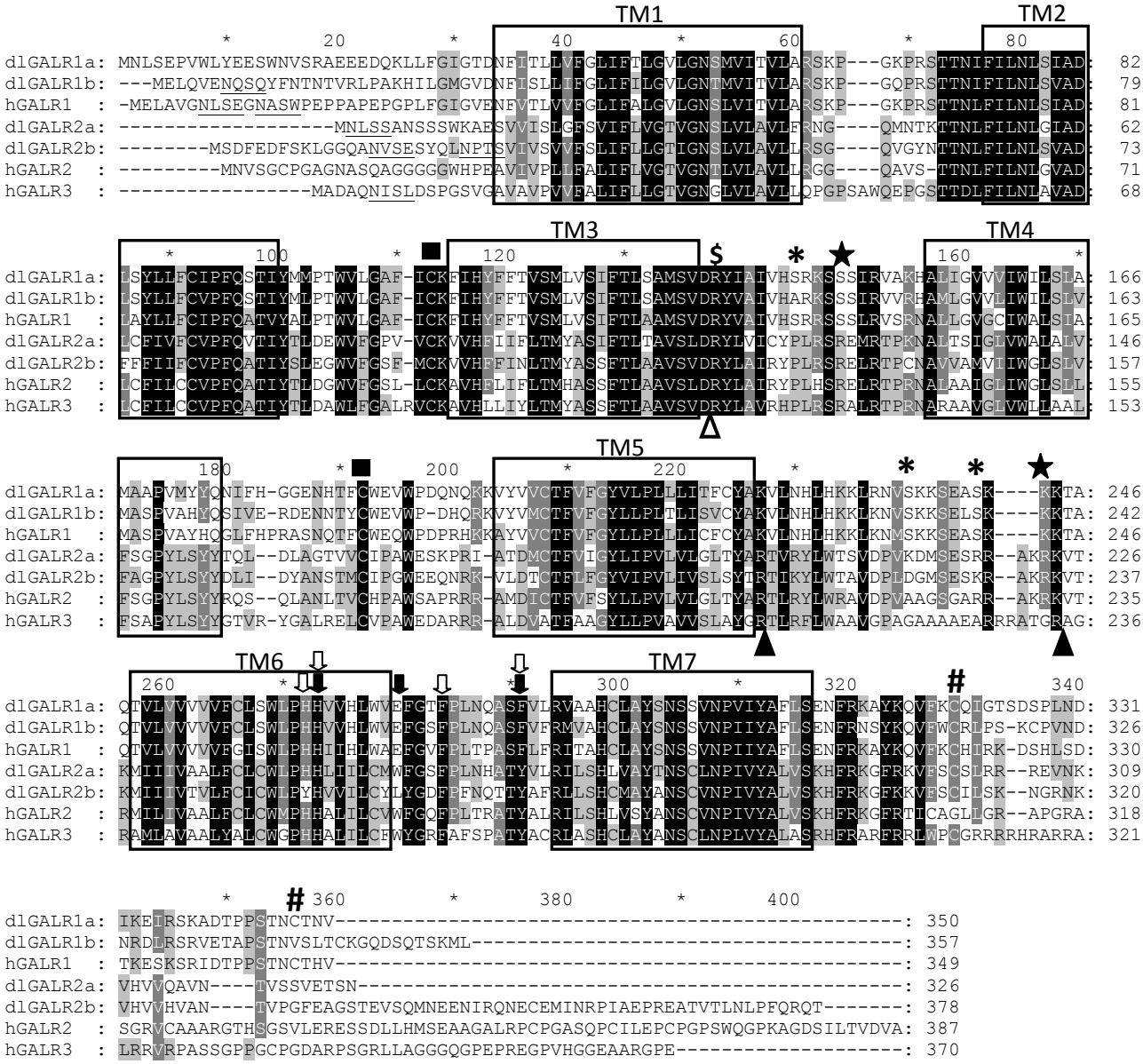
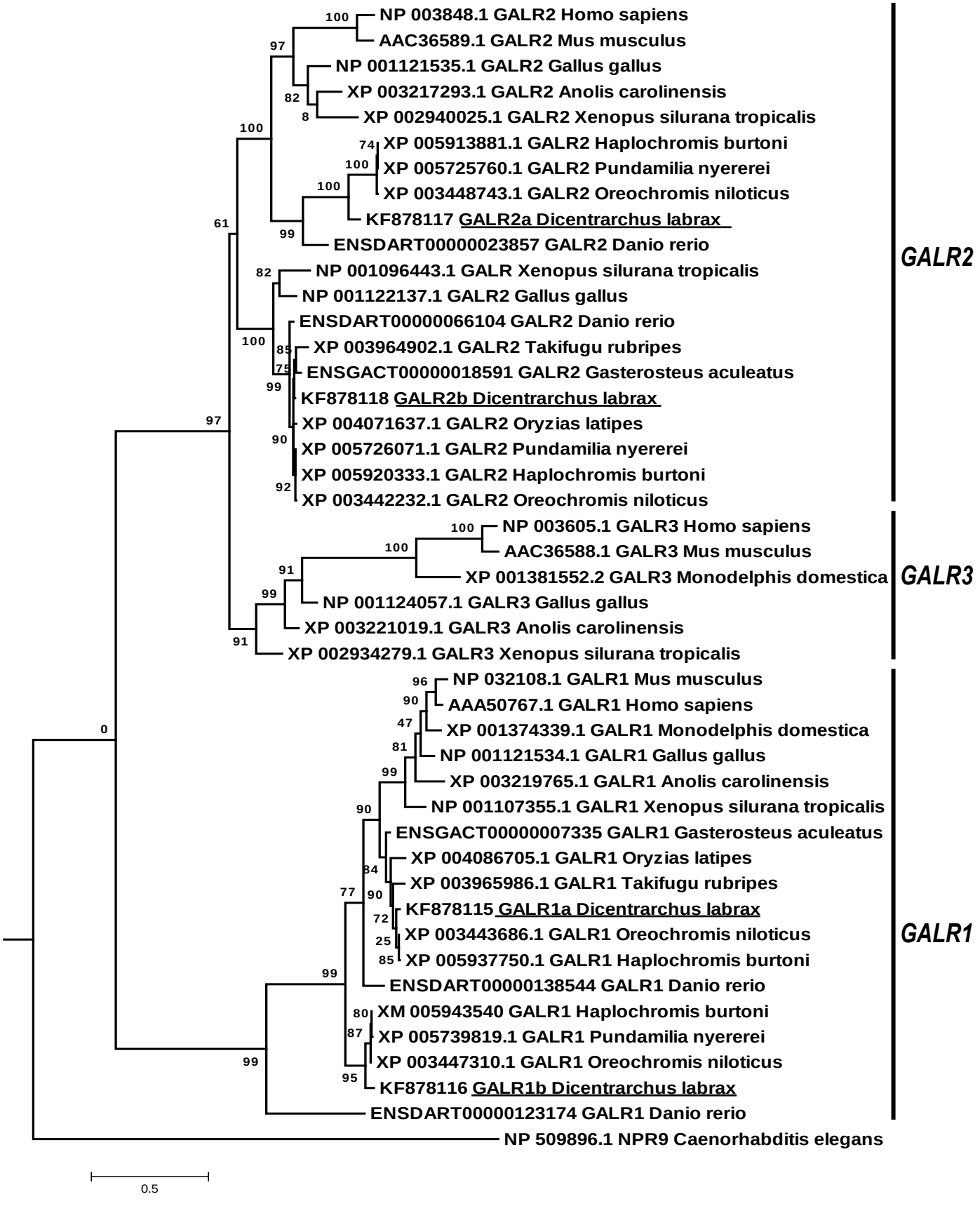

TM1
TM2
TM3
TM4
TM5
TM6
TM7
dlGALR1a: MNLSEPVWLYEESWNVSRAEEEDQKLLFGIGTDNFTTLLVFGLIFTLGVLGNSMVITVLARSKP---GKPRSTTNIFILNLSIAD: 82
dlGALR1b: ---MELQVENQSQYFNTNTVRLPAKHILGMGVDNFISLLIFGLIFILGVLGNTMVITVLARSKP---GQPRSTTNIFILNLSVAD: 79
hGALR1 : -MELAVGNLSEGNASWPEPPAPEPGPLFGIGVENFVTLVVFGLIFALGVLGNSLVITVLARSKP---GKPRSTTNLFILNLSIAD: 81
dlGALR2a: ----------MNLSSANSSSWKAESVVISLGFSVIFLVGTVGNSLVLAVLFRNG----QMNTKTTNLFILNLGIAD: 62
dlGALR2b: --------MSDFEDFSKLGGQANVSESYQLNPTSVIVSVVFSLIFLLGTIGNSLVLAVLLRSG----QVGYNTTNLFILNLSVAD: 73
hGALR2 : ---------MNVSGCPGAGNASQAGGGGGWHPEAVIVPLIFALIFLVGTVGNTLVLAVLLRGG----QAVS-TTNLFILNLGVAD: 71
hGALR3 : ----------------MADAQNISLDSPGSVGAVAVPVVFALIFLLGTVGNGLVLAVLLQPGPSAWQEPGSTTDLFILNLAVAD: 68
dlGALR1a: LSYLLFCIPFQSTIYMMPTWVLGAF-ICKFIHYFFTVSMLVSIFTLSAMSVDRYIAIVHSRKSSSIRVAKHALIGVVVIWILSLA: 166
dlGALR1b: LSYLLFCVPFQSTIYMLPTWVLGAF-ICKFIHYFFTVSMLVSIFTLSAMSVDRYVAIVHARKSSSIRVVRHAMLGVVLIWILSLV: 163
hGALR1 : LAYLLFCIPFQATVYALPTWVLGAF-ICKFIHYFFTVSMLVSIFTLAAMSVDRYVAIVHSRRSSSLRVSRNALLGVGCIWALSIA: 165
dlGALR2a: LCFIVFCVPFQVTIYTLDEWVFGPV-VCKVVHFIIFLTMYASIFTLTAVSLDRYLVICYPLRSREMRTPKNALTSIGLVWALALV: 146
dlGALR2b: FFFIIFCVPFQATIYSLEGWVFGSF-MCKVVHFFINLTMYASSFTLAAVSVDRYLAIRYPLRSRELRTPCNAVVAMVIIWGLSLV: 157
hGALR2 : ICFILCCVPFQATIYTLDGWVFGSL-LCKAVHFLIFLTMHASSFTLAAVSLDRYLAIRYPLHSRELRTPRNALAAIGLIWGLSLL: 155
hGALR3 : LCFILCCVPFQATIYTLDAWLFGALRVCKAVHLLIYLTMYASSFTLAAVSVDRYLAVRHPLRSRALRTPRNARAAVGLVWLLAAL: 153
dlGALR1a: MAAPVMYYQNIFH-GGENHTFCWEVWPDQNQKKVYVVCTFVFGYVLPLLLITFCYAKVLNHLHKKLRNVSKKSEASK----KKTA: 246
dlGALR1b: MASPVAHYQSIVE-RDENNTYCWEVWP-DHQRKVYVMCTFVFGYLLPLTLISVCYAKVLNHLHKKLKNVSKKSELSK----KKTA: 242
hGALR1 : MASPVAYHQGLFHPRASNQTFCWEQWPDPRHKKAYVVCTFVFGYLLPLLLICFCYAKVLNHLHKKLKNMSKKSEASK----KKTA: 246
dlGALR2a: FSGPYLSYYTQL--DLAGTVVCIPAWESKPRI-ATDMCTFVIGYLIPVLVLGLTYARTVRYLWTSVDPVKDMSESRR--AKRKVT: 226
dlGALR2b: FAGPYLSYYDLI--DYANSTMCIPGWEEQNRK-VLDTCTFLFGYVIPVLIVSLSYTRTIKYLWTAVDPLDGMSESKR--AKRKVT: 237
hGALR2 : FSGPYLSYYRQS--QLANLTVCHPAWSAPRRR-AMDICTFVFSYLLPVLVLGLTYARTLRYLWRAVDPVAAGSGARR--AKRKVT: 235
hGALR3 : FSAPYLSYYGTVR-YGALRELCVPAWEDARRR-ALDVATFAAGYLLPVAVVSLAYGRTLRFLWAAVGPAGAAAAEARRRATGRAG: 236
dlGALR1a: QTVLVVVVFCLSWLPHHVVHLWVEFGTFPLNQASFVLRVAAHCLAYSNSSVNPVIYAFLSENFRKAYKQVFKCQIGTSDSPLND: 331
dlGALR1b: QTVLVVVVFCLSWLPHHVVHLWVEFGSFPLNQASFVFRMVAHCLAYSNSSVNPIIYAFLSENFRNSYKQVFWCRLPS-KCPVND: 326
hGALR1 : QTVLVVVVFGISWLPHHIIHLWAEFGVFPLTPASFLFRITAHCLAYSNSSVNPIIYAFLSENFRKAYKQVFKCHIRK-DSHLSD: 330
dlGALR2a: KMIIIVAALFCLCWLPHHLIILCMWFGSFPLNHATYVLRILSHLVAYTNSCLNPIVYALVSKHFRKGFRKVFSCSLRR--REVNK: 309
dlGALR2b: KMIIIVTVLFCICWLPYHVVILCYLYGDFPFNQTTYAFRLLSHCMAYANSCVNPIVYALVSKHFRKGFKKVFSCILSK--NGRNK: 320
hGALR2 : RMILIVAALFCLCWMPHHALILCVWFGQFPLTRATYALRILSHLVSYANSCVNPIVYALVSKHFRKGFRTICAGLLGR--APGRA: 318
hGALR3 : RAMLAVAALYALCWGPHHALILCFWYGRFAFSPATYACRLASHCLAYANSCLNPLVYALASRHFRARFRRLWPCGRRRRHRARRA: 321
dlGALR1a: IKEIRSKADTPPSTNCTNV----------: 350
dlGALR1b: NRDLRSRVETAPSTNVSLTCKGQDSQTSKML----------: 357
hGALR1 : TKESKSRIDTPPSTNCTHV----------: 349
dlGALR2a: VHVVQAVN----TVSSVETSN----------: 326
dlGALR2b: VHVVHVAN----TVPGFEAGSTEVSQMNEENIRQNECEMINRPIAEPREATVTLNLPFQRQT-------: 378
hGALR2 : SGRVCAAARGTHSGSVLERESSDLLHMSEAAGALRPCPGASQPCILEPCPGPSWQGPKAGDSILTVDVA: 387
hGALR3 : LRRVRPASSGPPGCPGDARPSGRLLAGGGQGPEPREGPVHGGEAARGPE----------: 370

Figure 3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XP 005725760.1 GALR2 Pundamilia nyererei
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KF878117 GALR2a Dicentrarchus labrax
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NP 001096443.1 GALR Xenopus silurana tropicalis
NP 001122137.1 GALR2 Gallus gallus
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KF878118 GALR2b Dicentrarchus labrax
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XP 005726071.1 GALR2 Pundamilia nyererei
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XP 003442232.1 GALR2 Oreochromis niloticus
GALR2
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GALR3
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XP 003447310.1 GALR1 Oreochromis niloticus
KF878116 GALR1b Dicentrarchus labrax
ENSDART00000123174 GALR1 Danio rerio
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NP 509896.1 NPR9 Caenorhabditis elegans
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Figure4

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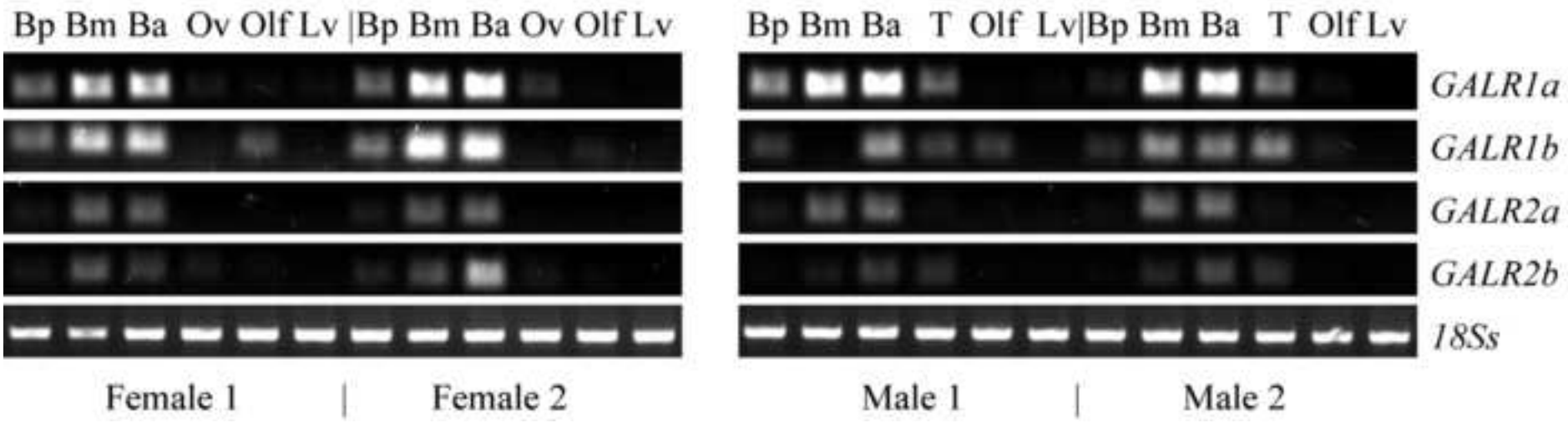


Figure 5

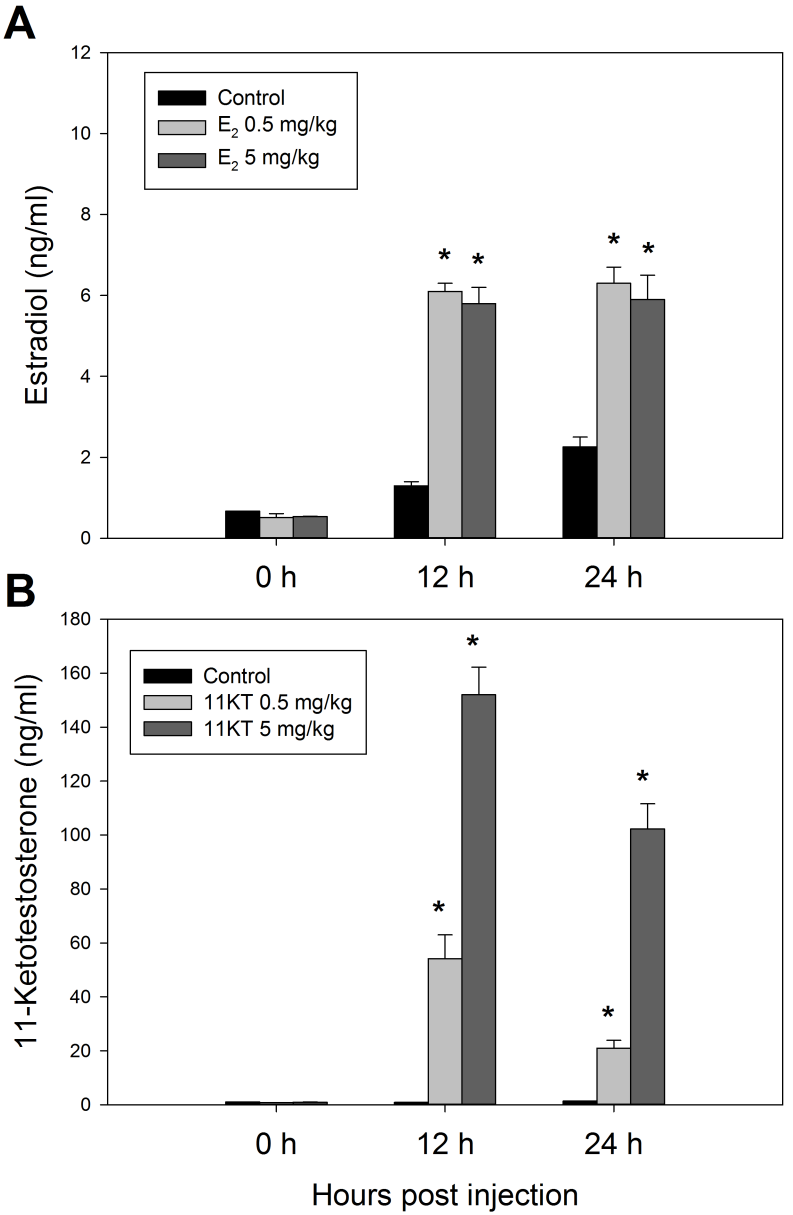

A
Estradiol (ng/ml)
Control
E2 0.5 mg/kg
E2 5 mg/kg
0 h
12 h
24 h
B
11-Ketotestosterone (ng/ml)
Control
11KT 0.5 mg/kg
11KT 5 mg/kg
0 h
12 h
24 h
Hours post injection

Figure6

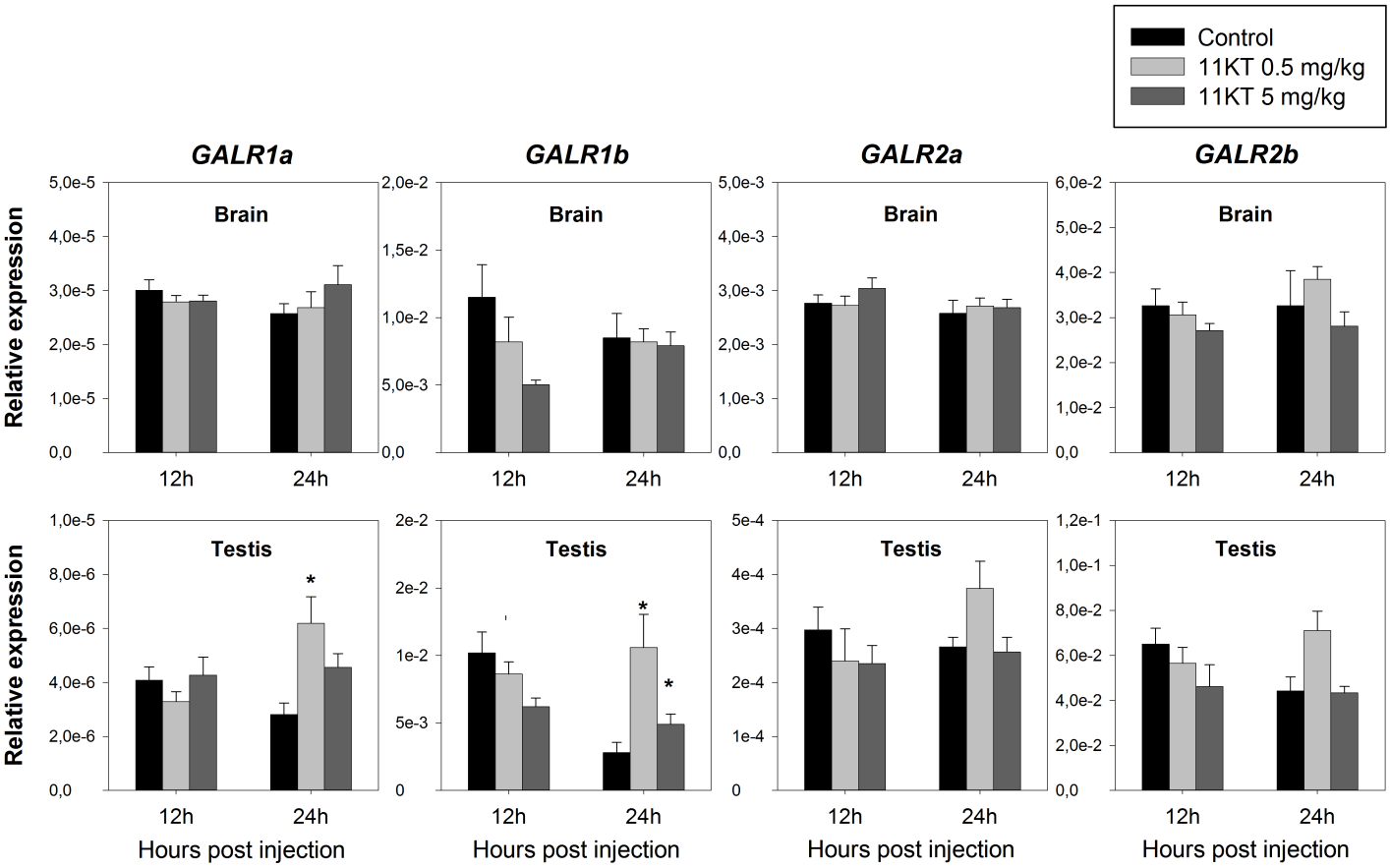
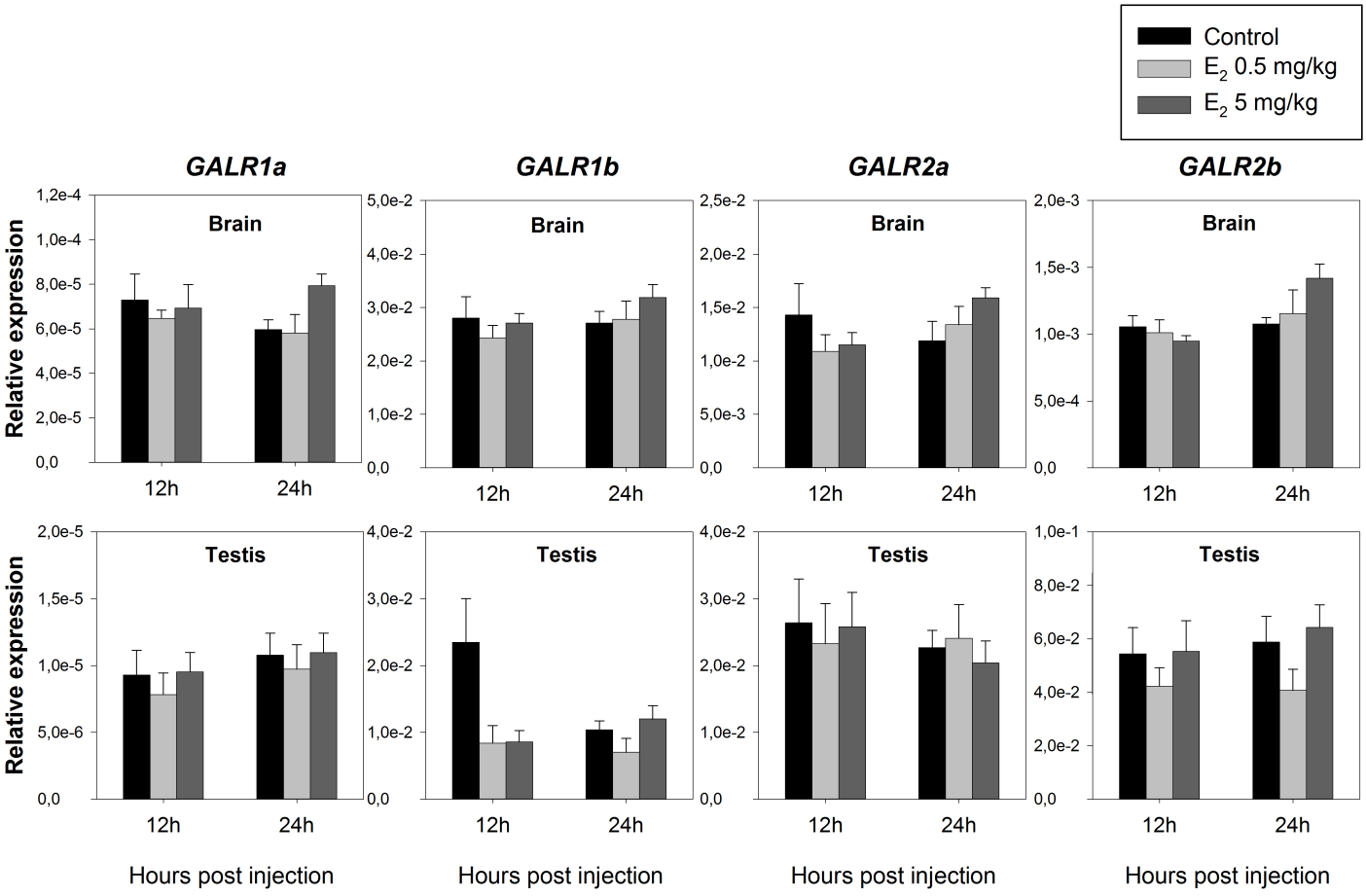

Control
11KT 0.5 mg/kg
11KT 5 mg/kg
GALR1a
GALR1b
GALR2a
GALR2b
Brain
Testis
Relative expression
12h
24h
Hours post injection
*

Figure7


Control
E2 0.5 mg/kg
E2 5 mg/kg
GALR1a
GALR1b
GALR2a
GALR2b
Brain
Testis
Relative expression
1,2e-4
1,0e-4
8,0e-5
6,0e-5
4,0e-5
2,0e-5
0,0
5,0e-2
4,0e-2
3,0e-2
2,0e-2
1,0e-2
2,5e-2
1,5e-2
5,0e-3
2,0e-3
1,5e-3
1,0e-3
5,0e-4
2,0e-5
1,5e-5
1,0e-5
5,0e-6
4,0e-2
3,0e-2
2,0e-2
1,0e-2
1,0e-1
8,0e-2
6,0e-2
4,0e-2
2,0e-2
12h
24h
Hours post injection

Supplementary Figure 1

Click here to download Supplementary Material: Supplementary Fig 1.docx