Characterisation and Expression of β1–, β2– and β3-Adrenergic Receptors in the Fathead Minnow (*Pimephales promelas*)

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Abstract

Complimentary DNAs for three beta-adrenergic receptors (βARs) were isolated and characterised in the fathead minnow. The encoded proteins of 402 (β_1AR), 397 (β_2AR) and 434 (β_3AR) amino acids were homologous to other vertebrate βARs, and displayed the characteristic seven transmembrane helices of G Protein-coupled receptors. Motifs and amino acids shown to be important for ligand binding were conserved in the fathead minnow receptors. Quantitative RT-PCR revealed the expression of all receptors to be highest in the heart and lowest in the ovary. However, the β_1AR was the predominant subtype in the heart (70%), and β_3AR the predominant subtype in the ovary (53%). In the brain, β_1AR expression was about 200-fold higher than that of β_2- and β_3AR, whereas in the liver, β_2AR expression was about 20-fold and 100-fold higher than β_3- and β_1AR expression, respectively. Receptor gene expression was modulated by exposure to propranolol (0.001 – 1 mg/L) for 21 days, but not in a consistent, concentration-related manner. These results show that the fathead minnow has a beta-adrenergic receptor repertoire similar to that of mammals, with the molecular signatures required for ligand binding. An exogenous ligand, the beta-blocker propranolol, is able to alter the expression profile of these receptors, although the functional relevance of such changes remains to be determined. Characterisation of the molecular targets for beta-blockers in fish will aid informed environmental risk assessments of these drugs, which are known to be present in the aquatic environment.

Keywords: Beta adrenergic receptor; β_1AR; β_2AR; β_3AR; fathead minnow; *Pimephales promelas*; G Protein-Coupled Receptor; gene expression, propranolol exposure.

1. Introduction

Adrenergic receptors (ARs) belong to the G protein-coupled receptor (GPCR) superfamily of proteins, which constitute the largest proportion of membrane signal transducers [38]. There are two main types of adrenoceptors, the αARs and βARs, and for each several subtypes have been identified in mammals: α_{1a}, α_{1b}, α_{1d}, α_{2a}, α_{2b}, α_{2c}, and β_1, β_2, β_3 [13, 59]. There is emerging evidence that fish also express the same receptors; for example, α_{1a}-, α_{1b}-, α_{1d} ARs have been characterised in rainbow trout [10]; α_{2b}-, α_{2c}- and α_{2d} ARs in zebrafish [54]; β_1-, β_2- and β_3 ARs in zebrafish [67] and black bullhead [16]; β_2- and β_3AR in rainbow trout [41, 42]. Additionally, genomic sequencing has identified homologues in medaka, stickleback, fugu and tetraodon, and a search of GeneBank reveals several partial sequences of teleost adrenoceptors.

The function of the adrenergic receptor system is believed to be the same in fish as it is in mammals, with activation of signal transduction following epinephrine/norepinephrine binding [19]. The recent high-resolution structural studies [11, 44, 46, 68] have provided experimental confirmation of the predicted molecular mechanism of GPCR activation in general, and of adrenoceptors in particular (reviewed by Rosenbaum et al., [52]). Our increased understanding of the structural requirements for receptor interaction and activation is useful when assessing the likelihood of receptors in other species becoming targets for agonists and antagonists designed for human receptors.

Our interest is the potential effects on aquatic organisms, especially fish, of pharmaceuticals present in the aquatic environment. Currently, approximately 150 different drugs have been detected in rivers and waterways [53] and there is concern that some of these, particularly those which have a high usage, are potent at low concentrations, poorly degraded or with a

propensity for bioaccumulation, may pose a threat to aquatic organisms [30, 61]. For example, ethinylestradiol, a component of the contraceptive pill, has been found to be a highly potent endocrine disrupter in fish at low environmental concentrations [8]. The beta-adrenoceptor blockers (β-blockers) are a group of pharmaceuticals widely prescribed for conditions such as high blood pressure, cardiac arrhythmias, glaucoma, anxiety and migraines, which exert their effects by binding to βARs and thereby preventing the interaction of epinephrine with its receptors. They are present in the aquatic environment at concentrations ranging from < 0.8 to 2900 ng/L [62, 65], and from acute EC_{50} data it appears that atenolol is non-toxic, whilst metoprolol would be classified as toxic and propranolol as very toxic to aquatic organisms [14]. Chronic data with respect to aquatic life and β-blockers are scarce, but recent studies indicate that these human drugs may affect fish at concentrations below toxic levels [43, 69]. This suggests the presence of βARs, and we report here the characterisation of three beta-adrenoceptors, β_1, β_2 and β_3, in the fathead minnow. The receptors contain the conserved amino acids and motifs identified as being important for agonist and antagonist binding, and receptor activation. Gene expression data also suggests that similar physiological effects to those seen in mammals may be expected following ligand binding, and modulation of receptor expression was seen following chronic exposure to propranolol. However, the functional importance of such changes requires information at the protein level, and remains to be determined.

2 Materials and Methods

2.1 Tissue acquisition

Fathead minnows, *Pimephales promelas*, were bred and maintained at Brunel University as detailed in Giltrow et al. [25]. Liver tissue was used for the characterisation of βARs, whilst receptor expression analysis was performed on liver, brain, heart and ovary tissue from

female fish. We also examined receptor expression in these tissues obtained from fathead minnows exposed to different concentrations of propranolol (0.001, 0.01, 0.1 and 1 mg/L) for 21 days [25]. The tissues were immediately snap frozen in liquid nitrogen after removal and stored at -80 °C until use.

2.2 Identification of βARs

Total RNA was extracted from liver using 1 ml of TriReagent (Sigma, Dorset, UK) for every 50 to 100 mg of tissue. The RNA quality was verified using 1.2 % agarose gel electrophoresis and quantified on a Nanodrop ND-1000 spectrophotometer. mRNA was isolated using Genelute mRNA miniprep kit (Sigma, Dorset, UK) and complementary DNA (cDNA) was obtained using Superscript III reagents and protocol (Invitrogen, Paisley, UK).

The oligonucleotide primers and annealing temperatures used in obtaining putative β_1AR, β_2AR and β_3AR fragments are shown in Table 1. AmpliTaq Gold (Applied Biosystems, Warrington, UK) was used in all PCR reactions. Putative βAR fragments were cloned, sequenced and localised to a particular βAR using Blast searches (www.ncbi.nlm.nih.gov). Rapid Amplification of cDNA Ends PCR (RACE PCR; Invitrogen, Paisley, UK and Clontech, California, USA) was used to obtain the remainder of the sequence in each direction. The complete receptor sequences were amplified using primers designed to the 3' and 5' untranslated regions (UTRs) and proof reading Taq (Pwo SuperYield DNA polymerase, Roche, Sussex, UK). Following cloning, the receptor sequences were confirmed by 'primer walking' in each direction in triplicate (Dundee University's Sequencing Service, www.dnaseq.co.uk).

2.3 Characterisation of βAR sequences

The fathead minnow βAR sequences were used to search for homologues in other species using Blast (www.ncbi.nlm.nih.gov). Sequence and phylogenetic analyses were performed using the following database entries: For β_1AR: *Homo sapiens* (human) NP_000675, *Danio rerio* (zebrafish) NP_001122161, *Takifugu rubripes* (fugu) ENSTRUP00000031392, *Gasterosteus aculeatus* (stickleback) ENSGACP00000008698, *Oryzias latipes* (medaka) ENSORLP00000006043, *Ovis aries* (sheep) AAB34523, *Mus musculus* (mouse) NP_031445, *Xenopus laevis* (frog) NP_001084152, *Meleagris gallopavo* (turkey) AAA49627; For β_2AR: Human AAA88015, zebrafish adrb2a NP_001096122, zebrafish adrb2b BAH84779, *Oncorhynchus mykiss* (rainbow trout) NP_001117912, *Tetraodon nigroviridis* (tetraodon) ENSTNIP00000020193, fugu AAQ02695, stickleback ENSGACP00000024398, medaka ENSORLP00000009383, sheep NP_001123626, mouse NP_031446, frog NP_001085791, *Ciona intestinalis* (ciona) XP_002121940; For β_3AR: Human NP_000016, zebrafish adrb3a BAH84778, zebrafish adrb3b NP_001128606, rainbow trout adrb3a NP_001118100, rainbow trout adrb3b NP_001117924, *Ameiurus melas* (black bullhead) adrb3b ABH10580, stickleback ENSGACP00000014582, fugu ENSTRUP00000020757, medaka ENSORLP00000014229, sheep AAG31167, mouse NP_038490; For β_{4c}AR: *Salmo salar* (salmon) NP_001133926, turkey AAA62150.

The positions of seven transmembrane helices were predicted using the hydropathy analysis programme TM_PRED (http://www.ch.embnet.org/software/TMPRED_form.html), and refined by manual comparisons to the crystal structures of bovine rhodopsin [44], turkey β_1AR [46] and human β_2AR [11, 68] in order to more accurately predict the length of the helices, as structural analysis had revealed these to extend further into the cytoplasm than suggested by hydropathy-based computer modelling. Prediction of palmitoylation sites was carried out using CSS-Palm 2.0 [47]. Potential phosphorylation of serine, threonine and

tyrosine residues in the intracellular loop 3 and cytoplasmic tail was identified using NetPhos 2.0, and protein kinase phosphorylation sites were predicted using NetPhosK 1.0 (http://www.cbs.dtu.dk/services/NetPhos) [6].

2. 4 Phylogenetic analysis

The βAR amino acid sequences were aligned using ClustalW (2.0.3), and the phylogenetic tree created using the Neigbourhood-Joining algorithm with 1000 bootstrap replicates in ClustalX (2.0.12) [36]. The un-rooted tree was visualised using Dendroscope V2.7.4 (www.dendroscope.org), and rooted with *Ciona intestinalis* β_2AR as the outgroup.

2.5 Gene Expression

The expression of β_1AR, β_2AR and β_3AR in liver, brain, ovary and heart of female fathead minnows was quantified using real-time PCR (QPCR). Primers were as follows: β_1AR (185bp) forward $^{5'}$CTTCGTATTTTTGAACTGGC$^{3'}$, reverse $^{5'}$CCATTGAGTTCACAAAGCCC$^{3'}$; β_2AR (224bp) forward $^{5'}$AGGTGATCAAGAGTCGAGTG$^{3'}$, reverse $^{5'}$ATGCTAATTAAGACCACCTC$^{3'}$; β_3AR (105bp) forward $^{5'}$GGCCCAGCAAAAAACATCC$^{3'}$, reverse $^{5'}$TTCCCATAGTGCTTGCCTCCTC$^{3'}$. The amplicons were cloned and sequenced to confirm their identities. QPCR standard βARs were prepared by in vitro transcribing cloned amplicons to generate RNA (Riboprobe, Promega), which was serially diluted (10^7-10^1 molecules) prior to use. Assays (20 μl) utilising Quantitect SYBR Green (Qiagen) and 0.5 μM (β_1AR and β_2AR) or 0.1 μM (β_3AR) each of forward and reverse primers were carried out in 96-well plates, with an efficiency greater than 90%. One μl of each sample mRNA (5 ng/μl), RNA standards and non-template control (sterile water) were assayed in triplicate. The QPCR cycling included a reverse transcription step (30 min at 50 ^{o}C, 15 min at 95 ^{o}C),

followed by 40 cycles of amplification (15 sec each at 95 °C, 55 °C and 72 °C for β_1AR; 15 sec each at 95 °C, 56 °C and 72 °C for β_2AR; 30 sec each at 95 °C, 55 °C and 72 °C for β_3AR), and elongation for 15 min at 72 °C. There was no significant difference between the threshold cycle (Ct) value for each RNA standard concentration between assay plates, and absolute gene expression was calculated from the standard curves and plotted as copies/ng mRNA.

3 Results

3.1 Sequence Analysis

The fathead minnow βAR sequences have GenBank accession numbers GQ901985 (β_1AR), GQ901986 (β_2AR) and GQ901987 (β_3AR). Fathead minnow cDNA sequences for β_1AR (1413 bp), β_2AR (1437 bp) and β_3AR (2203 bp) were found to code for proteins of 407, 397 and 434 amino acids, respectively (Fig. 1), which is comparable to the size of βARs in other species.

Blast searches showed fathead minnow β_1AR and β_2AR to be most homologous to zebrafish β_1AR and β_{2b}AR, respectively, with scores of 88% and 86% homology. Subsequent matches in these searches were to other fish and mammal β_1AR or β_2ARs. The fathead minnow β_3AR was found to be most homologous to zebrafish β_{3a}AR (74% homology), followed by other fish β_3AR and salmon β_{4c}AR receptors (around 60%). Identities to the human βARs are 51%, 55% and 40% for β_1AR, β_2AR and β_3AR, respectively. Comparison of the three fathead minnow receptors gave a homology of 51% between β_1AR and β_2AR, whereas β_3AR was 45% and 41% identical to β_1AR and β_2AR, respectively.

3.2 Topology and motifs

The positions and amino acid sequences of the seven transmembrane (TM) domains (Fig. 1) are highly conserved between different species; for example, fathead minnow and turkey β_1ARs have an average sequence identity of 79% (range 59-88%) in the TM regions compared to 57% over the whole sequence, and fathead minnow and human β_2AR have an average identity of 74% (range 57-86%) in the TMs compared to 58% overall. The TMs are also conserved between receptor types, and between the three fathead minnow βARs there is 53% identity in the TM regions compared to 31% overall.

In addition to the seven transmembrane helices, characteristic of all GPCRs, the fathead minnow βARs share motifs associated with the Family A (rhodopsin) receptors (Fig. 1). This family of GPCRs are characterised by a series of highly conserved residues in the TMs: $Asn^{1.50}$ in helix 1, $Asp^{2.50}$ in helix 2, $Arg^{3.50}$ in helix 3, $Trp^{4.50}$ in helix 4, $Pro^{5.50}$ in helix 5, $Pro^{6.50}$ in helix 6 and $Pro^{7.50}$ in helix 7 [23]. These fingerprint residues are numbered according to the Ballesteros-Weinstein numbering scheme, where the most conserved residue in each helix is given the number 50 in order to facilitate comparisons between different receptors [4], and all are present in the fathead minnow βARs. Most family A receptors also contain a disulfide bond connecting the beginning of TM3 and the second extracellular loop [23, 68], and in the fathead receptors this is proposed to be formed by $Cys^{3.25}$ and Cys202 (β_1), Cys194 (β_2) or Cys182 (β_3). The fathead minnow β_1AR Cys195-Cys201 and β_2AR Cys187-Cys193 are also likely to form an additional intra-loop disulfide bond in extracellular loop 2 [68], which is not present in β_3AR. Like most Family A receptors [23], the fathead minnow βARs are predicted to contain a palmitoylated cysteine in the carboxy-terminal tail at positions Cys 347 (β_1AR), Cys340 (β_2AR) and Cys349 (β_3AR). The amino-terminal region of the receptors has potential N-glycosylation sites (Asn-X-Ser/Thr), with three

223 possible sites in β_1AR and β_2AR. The β_3AR has one potential site in this region, but also has
224 the potential for glycosylation of extracellular loop 2 on Asn173.
225
226 Prediction of putative phosphorylation sites in the fathead minnow βARs revealed there to be
227 5, 6 and 10 potential Ser and Thr sites in the carboxy-terminal end of the β_1AR, β_2AR and
228 β_3AR, respectively. Additionally, intracellular loop 3 contained 2, 2 and 4 sites. Kinase
229 binding prediction suggested possible interaction with a range of kinases in all three
230 receptors, with highest scores obtained for protein kinase C in intracellular loop 3 of β_1AR
231 (Thr266) and β_3AR (Ser263), and protein kinase B with Ser247 of β_2AR. In the carboxy-
232 terminal tail, protein kinase C interaction was predicted for β_2AR (Ser361) and β_3AR
233 (Ser402).
234
235 **3.3 Phylogenetic analysis**
236 The phylogenetic analysis (Fig. 2) clearly differentiates the three beta-adrenergic receptor
237 subtypes. The fathead minnow and zebrafish are both members of the Cyprinidae family [66],
238 and this close evolutionary relationship is reflected in the grouping of their receptors.
239 Interestingly, the salmon β_{4c}AR showed close relatedness to the trout β_{3b}AR receptor, and
240 pair-wise alignment revealed these to be 97 % identical. It is therefore highly likely that the
241 salmon receptor is a β_3 subtype, and we propose it should be re-named accordingly. Turkey
242 β_{4c}AR is grouped with the human, mouse and sheep β_3ARs, but this receptor has been fully
243 characterised and shown to differ from the human β_3AR pharmacologically as well as
244 structurally [9].
245
246 **3.4 Gene expression**

Fathead minnow βARs were expressed in all tissues examined (Fig. 3), with highest expression observed in the heart (2,000-65,000 copies/ng mRNA) and lowest in the ovary (45-160 copies/ng mRNA). In the brain, expression of β_1AR was about 200-fold higher than that of β_2- and β_3AR, whilst in the liver the β_2AR was expressed about 20-fold and 100-fold higher than β_3AR and β_1AR, respectively. The expression of β_3AR showed the least variation in the tissues examined, with only about a 20-fold difference between lowest (10^2 copies/ng mRNA in brain and ovary) and highest ($2x10^3$ copies/ng mRNA in the heart) expression. In contrast, β_2AR expression varied about 350-fold between brain ($<10^2$ copies/ng mRNA) and heart ($2x10^4$ copies/ng mRNA), and β_1AR 1400-fold between ovary ($<10^2$ copies/ng mRNA) and heart ($6x10^4$ copies/ng mRNA).

Exposure of fathead minnows to propranolol for 21 days altered β_1-, β_2- and β_3AR expression in the different tissues, but not in a consistent, concentration-related manner (Fig. 4).

4 Discussion

We report here the characterisation of β_1-, β_2- and β_3ARs in the fathead minnow. The sequence comparisons and phylogenetic analyses provide unambiguous evidence that the three receptors represent different AR subtypes; the sequence identity between the three receptors range from 41% (β_1AR vs β_3AR) to 51% (β_1AR vs β_2AR), and they are located in different clades in the phylogenetic tree (Fig. 2). Comparison with the recently reported zebrafish receptors [67] shows that the fathead minnow β_2 receptor is most similar to the β_{2b}AR subtype (86% identity vs. 57% to the zebrafish β_{2a}AR), and the fathead minnow β_3 receptor is most similar to the β_{3a}AR (74% identity vs. 46% to zebrafish β_{3b}AR). However,

271 we were unsuccessful in obtaining other full-length β_2- and β_3ARs from fathead minnow
272 liver, and therefore believe it is inappropriate to refer to the receptors reported here as a
273 particular β_2- or β_3AR subtype. The presence of multiple copies of genes in teleost fish
274 would be expected, as three whole genome duplications (3R) are believed to have taken place
275 since the origin of vertebrates some 500 to 800 million years ago [21]. However, subsequent
276 gene loss has also occurred, and therefore there is uncertainty as to the number of gene
277 subtypes present in a single species. In a study designed to determine the number of βAR
278 genes in zebrafish [67], a single β_1AR and two β_2- and β_3AR genes were found. Given the
279 close relatedness between zebrafish and the fathead minnow, it is likely that there may be
280 further β_2- and β_3ARs encoded by the fathead minnow genome yet to be identified.
281 Phylogenetic analysis of 136 adrenoceptor sequences suggests that the βARs diverged 0.86-
282 0.37 billion years ago [3], and the chronogram presented by these authors implies that β_2AR
283 may have given rise to the β_1- and β_3ARs. In contrast, our analysis suggests β_1- and β_2ARs
284 to have evolved from a β_3AR, but this may be a function of the outgroup used (*Ciona*
285 *intesinalis* β_2AR) and the reduced number of sequences included in this analysis.
286
287 The fathead minnow receptors display the characteristic seven transmembrane helices of
288 GPCRs, and contain the conserved amino acid residues and motifs considered important for
289 ligand binding and activation. 3-D structural studies of human β_2AR and turkey β_1AR [46,
290 68] have helped identify residues important for receptor activation and signal transduction.
291 All three fathead minnow βARs have the amino acid residues believed to be important for
292 binding epinephrine in a human β_2AR [20], where interaction with the ligand occurs by the
293 formation of a salt bridge with $Asp^{3.32}$, by hydrogen bonding with $Ser^{5.42}$, $Ser^{5.43}$, $Ser^{5.46}$ and
294 $Asn^{6.55}$, and hydrophobic interactions with $Ile^{4.61}$ and $Phe^{6.52}$ (Ballesteros-Weinstein
295 numbering). All fathead minnow receptors also contain $Asn^{7.39}$, critical for the interaction

296 with antagonists such as propranolol [35], and $Val^{3.33}$, which by mutational analysis has been
297 shown to affect agonist and antagonist binding to hamster β_2AR [2]. The conserved DRY
298 motif in TM3 is thought to have a role in stabilising the inactive receptor conformation
299 through interaction with $Glu^{6.30}$ in TM6 [52], thereby maintaining a low constitutive activity
300 in the absence of ligand. The NPXXY motif at the end of TM7 is also believed to be
301 important in maintaining an inactive state through forming a water pocket network of
302 hydrogen bonds [52]. A further conserved residue, $Trp^{6.48}$, has been named the "rotamer
303 toggle switch" [57], due to its conformational transition required for GPCR activation.
304 Conservation of the important ligand binding residues suggests that the fathead minnow
305 receptors can interact with the same ligands as human βARs, including beta-blockers. This
306 conclusion is supported by Ruuskanen et al. [54], who found that ligand binding
307 characteristics, the order of potency and efficacy of tested agonists were all highly conserved
308 between zebrafish and human αARs.
309
310 Regulation of GPCRs, including the β-adrenergic receptor response, is primarily by
311 phosphorylation of serine or threonine residues in the third intracellular loop and C-terminal
312 tail by a broad range of protein kinases and G-protein coupled receptor kinases (GRK) [63].
313 This leads to receptor desensitisation, arrestin recruitment and receptor internalisation,
314 effectively terminating the signalling response. Previous studies of trout [41] and black
315 bullhead β_2ARs [16] indicated that fewer potential phosphorylation sites were present in fish
316 than in mammals, and this is believed to explain the apparent absence [15, 22] or reduced
317 effectiveness [16] of receptor desensitisation observed in physiological studies. The fathead
318 minnow β_3AR has a greater number of potential phosphorylation sites than predicted for β_1-
319 and β_2AR, as appears to also be the case for the black bullhead receptors [16]. However, the
320 significance of this is not known. Recent studies (reviewed by [32, 63]) have suggested that

the number of phospho-serine and phospho-threonine residues may not be the decisive factor in controlling GPCR signalling, as the extent of phosphorylation is not necessarily related to the affinity for arrestins, or subsequent internalisation. New roles for arrestins have also been discovered, whereby the internalised receptor-arrestin complex acts as a scaffold to facilitate protein-protein interactions, leading to activation of MAPK signalling cascades [45] and acting as a signal initiator rather than signal terminator. It is therefore clear that the regulation of the βARs may be complex, and further studies are required to elucidate the importance of the proposed phosphorylation sites.

The fathead minnow βARs were expressed in all tissues tested, with highest expression observed in the heart. There is general agreement that it is the β_1AR subtype which is of greatest functional importance in the heart [49], and similar to mammals the fathead minnow β_1AR receptors constitute 70% of the total βARs present in that tissue. The β_1AR is also the predominant subtype in zebrafish heart [67], and functional studies have shown that the heart rate of zebrafish [56] and medaka [31] can be modulated by isoproterenol (agonist) and propranolol (antagonist). Although these agents are not β_1AR specific [60], they would be anticipated to interact with the predominant βAR receptor present, and indeed *adrb1* morpholino knock-downs resulted in reduced heart rate [67]. β_3AR constitute only 2% of the fathead minnow heart adrenoceptors, and although β_3AR actions have been identified in rodent atria [58] and eel hearts [29] using isolated preparations, their physiological relevance in the presence of a much larger number of β_1ARs is not known. However, β_3ARs could have particular importance in specific areas of the heart, although in the human heart at least, this remains controversial [37].

345 Our study also indicates that β_1AR is the predominant receptor subtype (99%) in the fathead
346 minnow brain, in agreement with the high expression level seen in the brains of zebrafish
347 [67] and mammals [26]. The low expression of fathead minnow β_2AR in brain is similar to
348 the expression reported for the zebrafish β_{2b}AR, whereas the β_{2a}AR was highly expressed in
349 this tissue [67]. It is therefore likely that the distribution of β_2AR in zebrafish brain reported
350 by Ampatzis and Dermon [1] reflects the localisation of the β_{2a}AR subtype. The role of βARs
351 in the brain is likely to be diverse. For example, it has been suggested that activation of brain
352 βARs could have a role in energy metabolism, increasing glycogenolysis and Na^+/K^+-ATPase
353 activity in mice [27], and in mediating norepinephrine-modulated memory formation in the
354 chick [24].
355
356 In fathead minnow liver, the predominant receptor subtype is β_2AR (95%), which agrees
357 with the expression seen for zebrafish β_{2b}AR [67], rainbow trout [41] and mammal β_2AR
358 [48]. The metabolism of glucose and free fatty acids in liver is therefore likely to be mediated
359 via activation of this receptor [64].
360
361 Whilst there is general agreement with regards to tissue localisation of β_1AR and β_2ARs in
362 fish, differing localisation of β_3AR has been reported. In fathead minnow, the β_3AR was
363 detected in heart>liver>ovary>brain (this study), and additionally we have found it to be
364 present in the gill, red blood cell and adipose tissue (unpublished data). We therefore cannot
365 rule out that some of the β_3AR expression, especially in highly vascularised tissues such as
366 the heart and liver, is accounted for by residual blood in those tissues. Both zebrafish β_3ARs,
367 similar to trout β_{3b}AR, were expressed predominantly in the blood with very low expression
368 in other tissues [42, 67], whereas black bullhead β_{3b}AR was present in the liver [16], and

369 trout β_{3a}AR in heart, gill and red muscle [42]. The picture is further confused by the fact that
370 the trout β_3ARs share 84% sequence identity and are more similar to zebrafish β_{3a}- than β_{3b}
371 receptors. The apparent broad tissue distribution of fish β_3AR is similar to mammals, where
372 β_3 receptors have been found to be present not only in adipose tissue, but also in liver,
373 muscle [17] gallbladder, colon [34], brain [50], stomach, prostate [5] and oocytes [12], with
374 the possibility of wide-ranging actions. Interestingly, whilst expression levels in the fathead
375 minnow ovary were low, the β_3AR was the predominant adrenoceptor (53%) in that tissue. A
376 specific role for the β_3AR in the human myometrium has been suggested by a study showing
377 that it is the predominant βAR subtype in that tissue, and it is upregulated in near-term
378 pregnant myometrium [51].
379
380 Exposure to propranolol, a non-selective beta-blocker, altered expression of the three βARs
381 to different extents in the fathead minnow tissues tested, and whilst some significant up- and
382 down-regulation (compared to control fish) were observed, there were no consistent dose-
383 response effects. The lack of a concentration-related response is particularly interesting given
384 that at the highest (0.1 and 1 mg/L), but not the lower (0.001 and 0.01 mg/L) doses, plasma
385 propranolol levels in the fish exceeded human therapeutic levels [25]. Studies in man and rats
386 are conflicting, but suggest that chronic treatment with beta-blockers can up-regulate receptor
387 density [7], although there appears to be no direct relationship between receptor gene
388 expression and protein levels [28, 39]. However, it should be noted that the human data is
389 generally obtained from studies of people with severe cardiac problems, and the response
390 may therefore not be comparable to our study with healthy fish. Thus, whilst it appears that
391 chronic exposure to propranolol can modulate βAR gene expression in fathead minnow
392 tissues, the results need to be replicated and include receptor protein measurements and
393 functional data in order to interpret these changes in a meaningful way.

In summary, we have shown the presence of three beta adrenergic receptor subtypes (β_1, β_2, β_3) in the fathead minnow. The conserved amino acid residues and motifs important for agonist and antagonist binding are present, and tissue localisation of the receptors is similar to that observed in humans. It is therefore likely that pharmaceuticals targeting these receptors could cause effects if present in the aquatic environment, but further work is required to determine if such effects are detrimental at environmental concentrations. Our studies to date would suggest levels would need to reach mg/L concentrations; acute toxicity has only been observed at very high exposure concentrations of atenolol (>10 mg/L) [69] and propranolol (>3 mg/L) [25], and reproductive effects at 1 mg/L propranolol [25]. We also have an indication that beta-adrenergic receptor specific actions, such as the effect of propranolol on heart rate, require exposure to mg/L concentrations [41]. Although the environmental concentration of propranolol is generally in the ng/L range, levels as high as 6.5 μg/L have been reported in hospital effluents in Spain [55]. The latter is still an order of magnitude below where effects have been observed, suggesting that, in general, propranolol will not constitute an environmental hazard. However, the propensity for propranolol to bioaccumulate [18, 25] indicates that, in some specific locations, it could be of concern.

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614 Figure Legends

615

616 Figure 1. Alignment (Clustal 2.2.10) of fathead minnow β_1-, β_2- and β_3AR amino acid
617 sequences. Transmembrane helices (TM1-TM7) are indicated below the alignment. Light
618 grey boxes indicate putative N-linked glycosylation sites. Residues in *squares* are those
619 highly conserved throughout the rhodopsin family of GPCRs. Cysteines involved in disulfide
620 bonding are shown in *green*. Ligand binding residues (*red*) and motifs important in stabilising
621 the inactive receptor (*light blue*) are shown. The "rotamer toggle switch" is shown in *yellow*.
622 The palmitoylation site is shown in *dark blue*. Potential phosphorylation sites (*dark grey*) are
623 indicated. (*) Indicates identical amino acid sequence in that column, (**:**) shows conserved
624 substitutions have been made and (**.**) indicates that semi-conserved substitutions are observed.

625

626 Figure 2. Phylogenetic tree constructed using the neighbour-joining algorithm, with *Ciona*
627 *intestinalis* β_2AR as the outgroup. The node numbers refer to bootstrap values after 1000
628 iterations. The β_1- (adrb1), β_2- (adrb2) and β_3 adrenergic receptors (adrb3) are present in
629 different clades, and the fathead minnow βARs are grouped with the respective zebrafish
630 receptors.

631

632 Figure 3. mRNA expression of β_1-, β_2- and β_3 adrenergic receptors in female fathead
633 minnow tissues. Using RNA standards, absolute levels of expression was determined and
634 data is presented as number of copies/ng mRNA +/-SEM. Three replicates of each sample
635 were analysed; n=7-8 for liver, ovary and brain, whereas the small size of the heart required
636 these to be pooled and as a consequence n=1.

637

638 Figure 4. mRNA expression of β_1-, β_2- and β_3 adrenergic receptors in female fathead
639 minnow tissues obtained from fish exposed to different concentrations of propranolol (0,
640 0.001, 0.01, 0.1 and 1 mg/L) for 21 days. Propranolol concentrations were measured and
641 found to be similar to nominal values [25]. Using RNA standards, absolute levels of
642 expression was determined and data is presented as number of copies/ng mRNA +/-SEM.
643 Three replicates of each sample were analysed; n=7-8 for liver, ovary and brain, whereas the
644 small size of the heart required these to be pooled and as a consequence n=1.

Table 1. Gene specific primers used for amplification of fragments and complete sequences of the β_1-, β_2- and β_3ARs in the Fathead Minnow

Receptor	**Forward Primer**	**Reverse Primer**	**Annealing Temperature ^{o}C** [a]
β1-AR initial fragments	$^{5'}$GACTCTAAACGCGCCACG$^{3'}$ $^{5'}$CCCCATCCTAATGCACTGG$^{3'}$	$^{5'}$CAATTACGCACAGGGTCTCG$^{3'}$ $^{5'}$AGCCTTCTGCTCTTTAAAGC$^{3'}$	62.0 56.1
β1-AR 3' RACE[b] (nested reactions)	GSP1: $^{5'}$GACACCGTGGATACATCATGCTATAACG$^{3'}$ GSP2: $^{5'}$CCAGGGTATACAGAGAAGCCAAACAACAACTG$^{3'}$ GSP3: $^{5'}$GCAAACCTAACCGAAAACGAACCAC$^{3'}$		67.0, 66.0, 65.0 70.6, 68.5, 65.0 67.5, 66.0, 65.0
β1-AR 5' RACE[b]		$^{5'}$ATCAAGAGATATCCAGAATTCACAGAAGAACGA$^{3'}$	67.0, 65.0, 60.0
β1-AR whole sequence	$^{5'}$GAGAGCGCGGATGGAAG$^{3'}$	$^{5'}$GGAAATATTTTCGAATTTGTCTGAAACG$^{3'}$	58.9
β2-AR initial fragments	$^{5'}$CTRGTKMTRKKCATWGTCTTTGG$^{3'}$ $^{5'}$GTACGTCGCCATCATGTGG$^{3'}$	$^{5'}$CACACCSYYAYSACCAYCMCGCA$^{3'}$ $^{5'}$GTTGTCCAYCTTCCAGATGGYYR$^{3'}$	56.1 62.0
β2-AR 3' RACE[b] (nested reactions)	GSP1: $^{5'}$CAGGACGGGAACGAGACGAAGAAC$^{3'}$ GSP2: $^{5'}$GACCACAAAGCTCTGAAGACCTTGGG$^{3'}$ GSP3: $^{5'}$AACATTCACCCTCTGCTGGCTGC$^{3'}$		69.5, 67.0, 65.0 69.0,67.0, 65.0 69.0, 67.0, 65.0
β2-AR 5' RACE[b] (nested reactions)		GSP1: $^{5'}$CAAAACTCGCAGAAGAAGTTTCCGAAGTG$^{3'}$ GSP2: $^{5'}$CTGATGAAGTAGTTGGTGCCCGTCTG$^{3'}$ GSP3: $^{5'}$GTTGAAATCGTACAATGGCGCTGATGAC$^{3'}$	63.0 63.0 70.7, 68.0,65.0
β2-AR whole sequence	$^{5'}$CGACATTTAGTCTACAGCCGAGAGTG$^{3'}$	$^{5'}$ACATCTAAAAACCATGTTTTGTCACAGAC$^{3'}$	54.0
β3-AR initial fragments (nested reactions)	1: $^{5'}$GTAACCTCCTGGTCATCATTG$^{3'}$ 2: $^{5'}$CCTCCAGCTGCAGACTAC$^{3'}$ 3: $^{5'}$GCCAGCATAGAGACTCTATG$^{3'}$	1: $^{5'}$GTAGATGATAGGGTTGAGTCC$^{3'}$ 2: $^{5'}$GCCTAACCAGTTTAAGAGACG$^{3'}$ 3: $^{5'}$GCGTAGATGATGTTCGCCAC$^{3'}$	62.0 62.0 64.0
β3-AR 3' RACE[b]	GSP1: $^{5'}$TTCAATCGAGATCTGCTAACC$^{3'}$		68.0

(nested reactions)	GSP2: $^{5'}$CAGAGTTTCGTGCGGCCTT$^{3'}$		68.0
β3-AR 5' RACE[b] (nested reactions)		GSP1: $^{5'}$CGATGTCCATAATCTGCACG$^{3'}$ GSP2: $^{5'}$GGTCTGCAGATGGGAGGT$^{3'}$	68.9 69.6
β3-AR whole sequence	$^{5'}$CACGCTGACTGAACCTCCTCC$^{3'}$	$^{5'}$CCGGGCTGAAGTGGATTGCTCCAGTAC $^{3'}$	70.0

[a]The three temperatures denotes those used for cycles 1-5 cycles, cycles 6-10 and cycles 11- 30, respectively. [b]For 3' and 5' RACE the reverse and forward primers were those provided by the manufacturer.

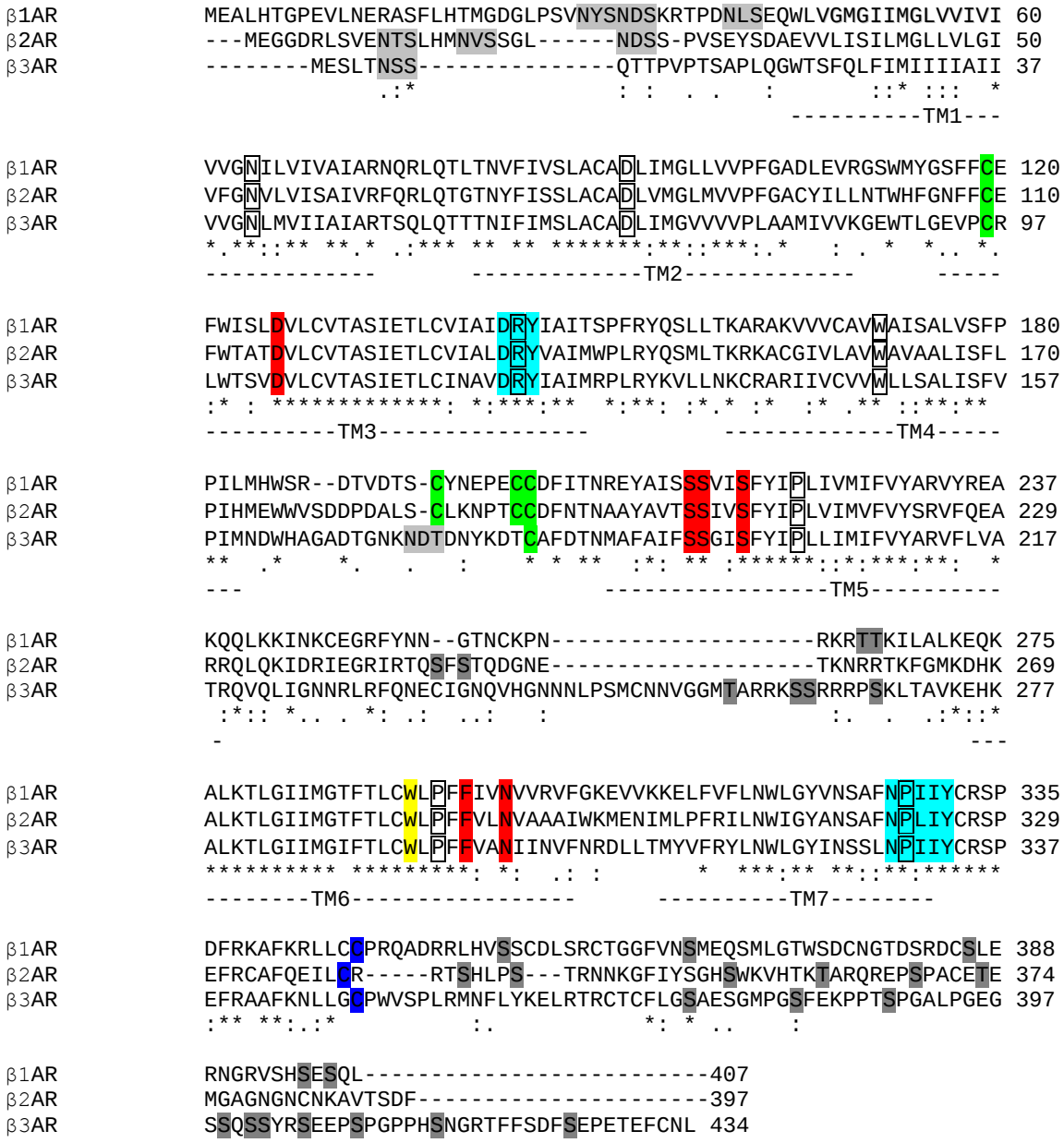
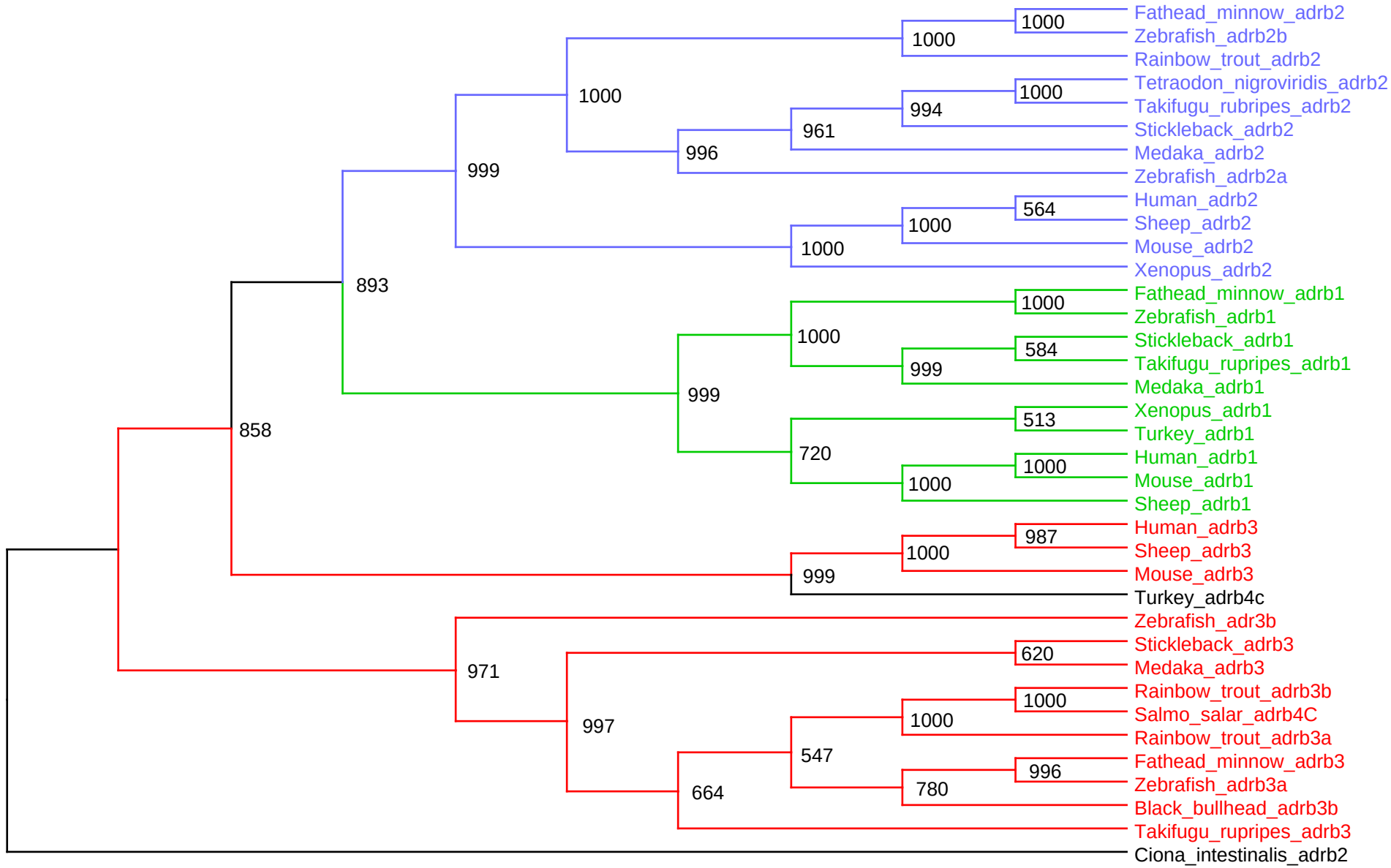

β1AR MEALHTGPEVLNERASFLHTMGDGLPSVNYSNDSKRTPDNLSEQWLVGMGIIMGLVVIVI 60
β2AR ---MEGGDRLSVENTSLHMNVSSGL------NDSS-PVSEYSDAEVVLISILMGLLVLGI 50
β3AR --------MESLTNSS--------------QTTPVPTSAPLQGWTSFQLFIMIIIIAII 37
TM1
β1AR VVGNILVIVAIARNQRLQTLTNVFIVSLACADLIMGLLVVPFGADLEVRGSWMYGSFFCE 120
β2AR VFGNVLVISAIVRFQRLQTGTNYFISSLACADLVMGLMVVPFGACYILLNTWHFGNFFCE 110
β3AR VVGNLMVIIAIARTSQLQTTTNIFIMSLACADLIMGVVVVPLAAMIVVKGEWTLGEVPCR 97
TM2
β1AR FWISLDVLCVTASIETLCVIAIDRYIAITSPFRYQSLLTKARAKVVVCAVWAISALVSFP 180
β2AR FWTATDVLCVTASIETLCVIALDRYVAIMWPLRYQSMLTKRKACGIVLAVWAVAALISFL 170
β3AR LWTSVDVLCVTASIETLCINAVDRYIAIMRPLRYKVLLNKCRARIIVCVVWLLSALISFV 157
TM3 TM4
β1AR PILMHWSR--DTVDTS-CYNEPECCDFITNREYAISSSVISFYIPLIVMIFVYARVYREA 237
β2AR PIHMEWWVSDDPDALS-CLKNPTCCDFNTNAAYAVTSSIVSFYIPLVIMVFVYSRVFQEA 229
β3AR PIMNDWHAGADTGNKNDTDNYKDTCAFDTNMAFAIFSSGISFYIPLLIMIFVYARVFLVA 217
TM5
β1AR KQQLKKINKCEGRFYNN--GTNCKPN-------------------RKRTTKILALKEQK 275
β2AR RRQLQKIDRIEGRIRTQSFSTQDGNE-------------------TKNRRTKFGMKDHK 269
β3AR TRQVQLIGNNRLRFQNECIGNQVHGNNNLPSMCNNVGGMTARRKSSRRRPSKLTAVKEHK 277
TM6 TM7
β1AR ALKTLGIIMGTFTLCWLPFFIVNVVRVFGKEVVKKELFVFLNWLGYVNSAFNPIIYCRSP 335
β2AR ALKTLGIIMGTFTLCWLPFFVLNVAAAIWKMENIMLPFRILNWIGYANSAFNPLIYCRSP 329
β3AR ALKTLGIIMGIFTLCWLPFFVANIINVFNRDLLTMYVFRYLNWLGYINSSLNPIIYCRSP 337
TM8
β1AR DFRKAFKRLLCCPRQADRRLHVSSCDLSRCTGGFVNSMEQSMLGTWSDCNGTDSRDCSLE 388
β2AR EFRCAFQEILCR-----RTSHLPS---TRNNKGFIYSGHSWKVHTKTARQREPSPACETE 374
β3AR EFRAAFKNLLGCPWVSPLRMNFLYKELRTRCTCFLGSAESGMPGSFEKPPTSPGALPGEG 397
β1AR RNGRVSHSESQL-------------------------407
β2AR MGAGNGNCNKAVTSDF----------------------397
β3AR SSQSSYRSEEPSPGPPHSNGRTFFSDFSEPETEFCNL 434

Figure 1.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Zebrafish_adrb2b
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Medaka_adrb2
Zebrafish_adrb2a
Human_adrb2
Sheep_adrb2
Mouse_adrb2
Xenopus_adrb2
Fathead_minnow_adrb1
Zebrafish_adrb1
Stickleback_adrb1
Takifugu_rupripes_adrb1
Medaka_adrb1
Xenopus_adrb1
Turkey_adrb1
Human_adrb1
Mouse_adrb1
Sheep_adrb1
Human_adrb3
Sheep_adrb3
Mouse_adrb3
Turkey_adrb4c
Zebrafish_adr3b
Stickleback_adrb3
Medaka_adrb3
Rainbow_trout_adrb3b
Salmo_salar_adrb4C
Rainbow_trout_adrb3a
Fathead_minnow_adrb3
Zebrafish_adrb3a
Black_bullhead_adrb3b
Takifugu_rupripes_adrb3
Ciona_intestinalis_adrb2
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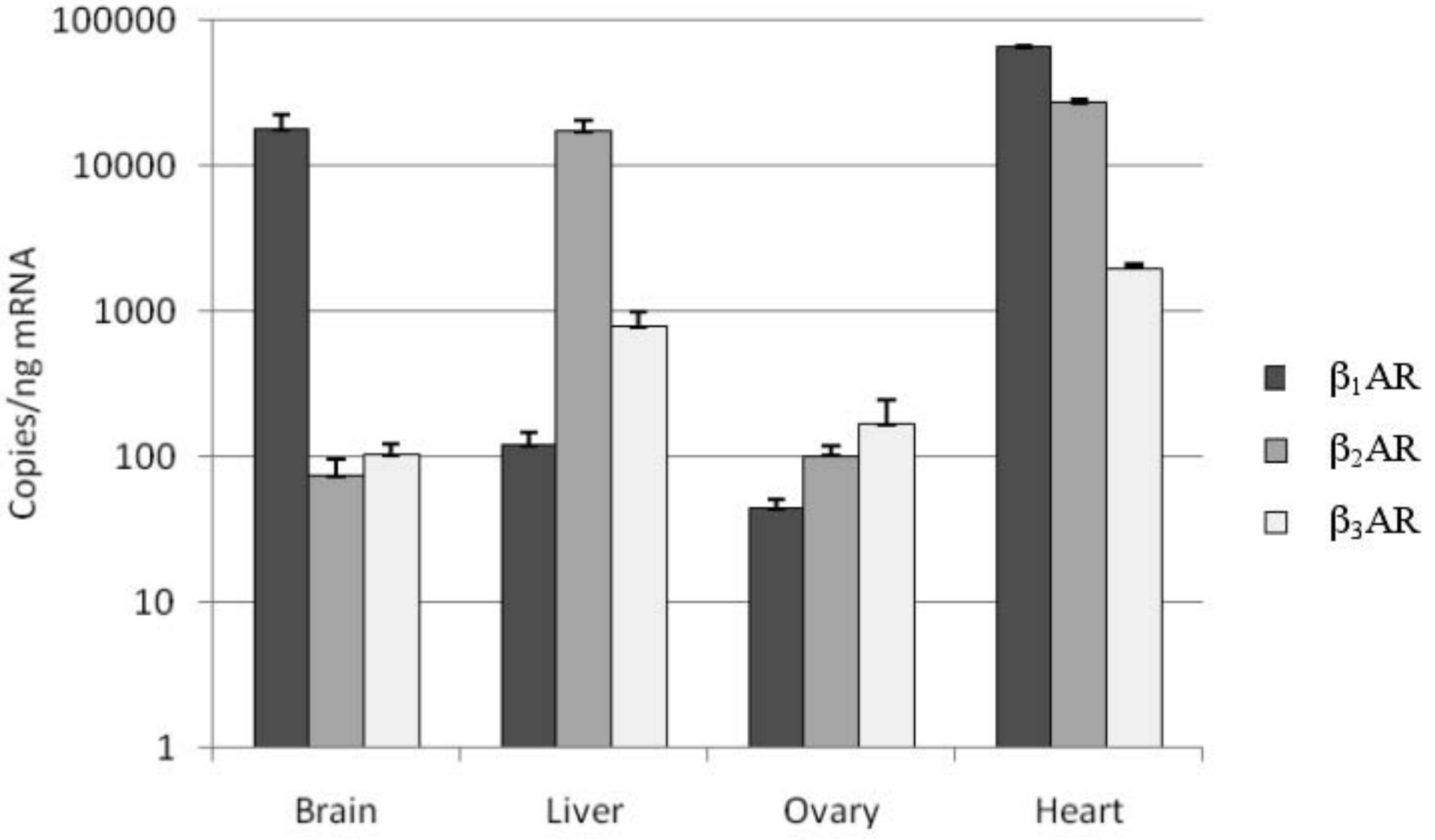
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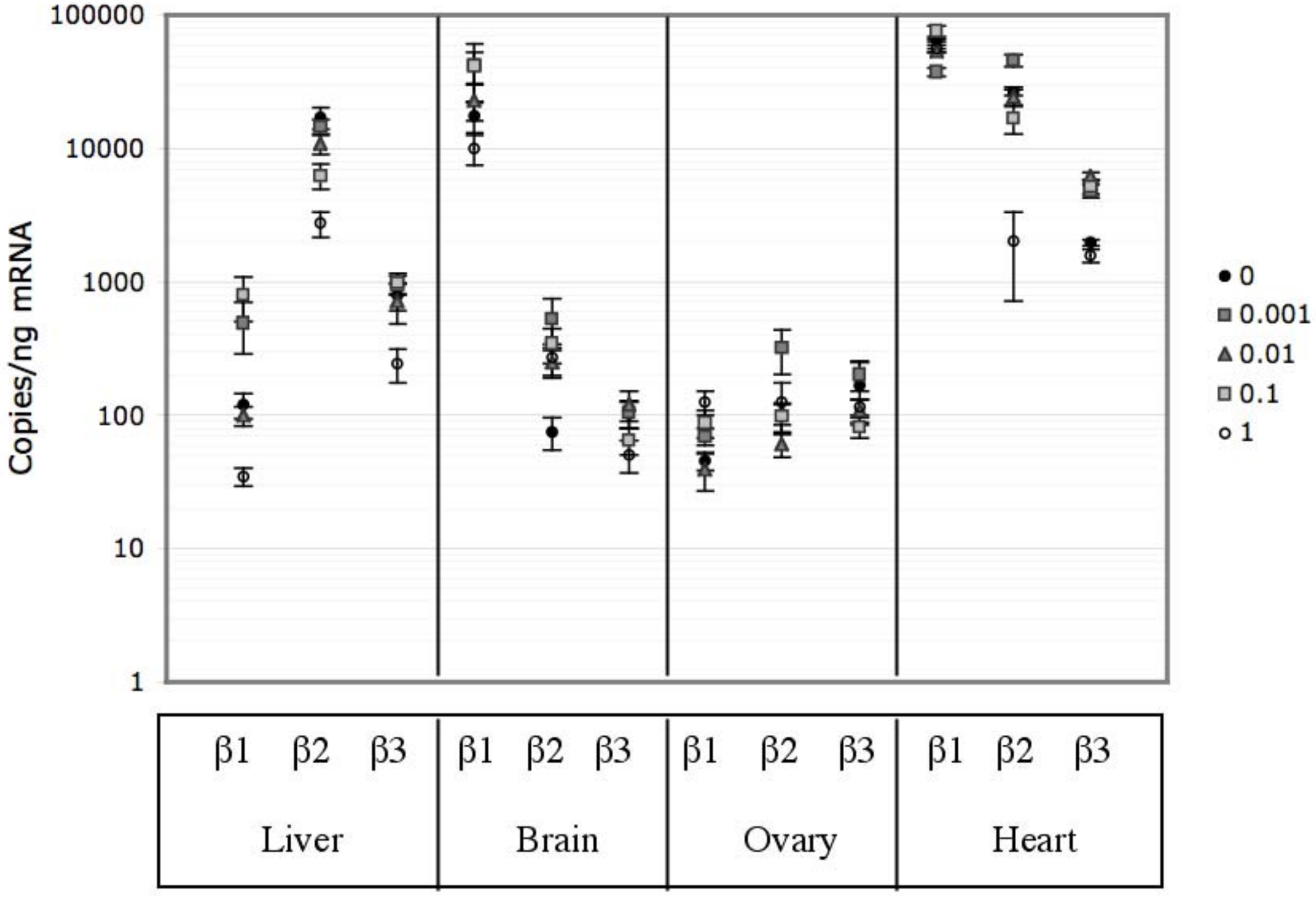
Brain
Liver
Ovary
Heart
β1AR
β2AR
β3AR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β1 β2 β3
Liver
β1 β2 β3
Brain
β1 β2 β3
Ovary
β1 β2 β3
Heart