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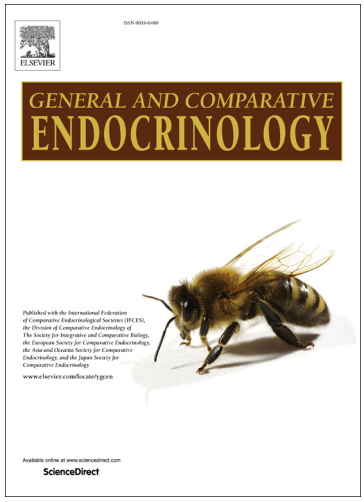

The evolution, structure and function of the ray finned fish (Actinopterygii) glucocorticoid receptors

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Abstract

Basal ray-finned fish (Actinopterygii) possess a single glucocorticoid receptor (GR) and when compared to the lobe-finned vertebrate (Sarcoptergyii) GR possess nine additional amino acids between the zinc-finger of the DNA binding domain. A whole genome duplication event which occurred between 320-350 MYA in the teleost lineage following the split from the basal ray-finned fish resulted in 2 GRs: one GR group, GR1, has retained the 9 amino acids insert whereas the other group, GR2, has not. The exception to this is the zebrafish, that have lost one of the GRs, but they do possess 2 GRs with a splice variant that lacks the C-terminal portion of the GR to form GRβ which acts as a dominant-repressor of the wildtype GR. Another splice variant sees the basal ray-finned GR and teleost GR1 without the 9 amino acids insert. The molecular basis for GRs retention is beginning to be unravelled. In *Pantadon buchholzi*, rainbow trout, carp, marine and Japanese medaka GR2 is more sensitive to glucocorticoids (GC), thus potentially playing a more significant role in regulating gene expression at basal circulatory GC concentrations. However, this division in GC sensitivity is not seen in other species. The few studies to evaluate the significance of the 9 amino acid insert have shown that it affect maximal transactivational activity the extent to which is dependent on the number of glucocorticoid response elements (GREs) present in the reporter plasmid. The retention of these GRs would suggest there was an evolutionary advantage, which saw the development of a complex regulatory process to mediate the actions of the glucocorticoids.

1. Introduction

The corticosteroid receptors (CR), which include the glucocorticoid (GR) and mineralocorticoid receptors (MR), belong to the nuclear receptor family of proteins. The genes that encode for these proteins evolved from a common ancestor steroid receptor (SR) present in the chordates, following rounds of whole genome or gene duplication events, with the CRs emerging in the vertebrate lineage approximately 500MYA. A further whole genome

duplication event in the teleost lineage between 320 – 350 MYA has given rise to 2 GR isoforms (Figure 1). The majority of functional and structural analysis has been carried out on the tetrapod GRs and these studies have been used to compare and contrast the properties of the basal actinopterygian (ray-finned fish) GRs and the teleost GRs (Arterberry et al 2012, Becker et al 2008, Bury et al 2003, Ducouret et al 1995, Greenwood et al 2003, Kim et al 2011, Li 2012, Miyagawa et al 2014, Oka et al 2015, Stolte et al 2008, Sturm et al 2005, Sturm et al 2010, Sturm et al 2011). This short review will first describe the structure and function of the human GRs, and then briefly describe the evolution of the steroid receptors, that gave rise to the actinopterygian GRs. Finally, the structure and function of the two teleost GRs will be discussed and potential reasons for their retention proposed.

2. Glucocorticosteroid receptor structure and function

The gene encoding for the human GR is composed of 9 exons (Oakley and Cidlowski, 2011) and the translated protein is described as having 4 functional regions. The protein contains a highly conserved central DNA-binding domain (DBD) or C-domain, encoded on exon 3 and 4, which contains two zinc-fingers and recognises specific palindromic DNA sequences of the glucocorticoid response element (GRE) upstream of the target genes, and is also a region important for homodimer formation. The hormone binding domain (HBD), or E-domain, and hinge region, or D-domain, are encoded on exons 5 – 9. The C-terminus HBD is also highly conserved between GRs, specifically the 22 amino acids that interact with the glucocorticoid hormones, to form the hydrophobic ligand binding pocket that characterises all GRs (Bledsoe et al 2002). The HBD is also the sight of two transactivation functional sites, named activation function 2 (AF2) and τ2 (Hollenberg and Evans 1988; Kucera et al 2002). The D-domain, located between the DBD and HBD, is the least conserved region and is involved in protein folding. Additionally, one of two nuclear localisation signals, NL1, spans the DBD/hinge region transition; the other, NL2, is present in the HBD (Bamberger et al 1996). The N-terminal transactivation domain (NTD) also known as the A/B domain, is encoded on exon 2; it is also not well conserved between the vertebrate GR, but is the site

of the ligand-independent AF1 site (Giguere et al 1986, Oka et al 2015, Sturm et al 2011). Classically, the inactive GRs reside in the cytoplasm as part of a large heteromeric complex which includes HSP90 and immunophilins (Heitzer et al 2007). Following ligand/receptor binding, the GR dissociates and is transferred to the nucleus where it forms homodimers, interacts with GREs and stimulates gene expression. Alternately, the GR-ligand complex may interact with less well defined negative GREs to suppress gene expression. The GRs can also interact with other transcription factors to repress or enhance gene expression (Glass and Rosenfeld, 2000).

However, alternate splice variants, different translation initiation sites, post-translation modifications (e.g. phosphorylation) and single nucleotide polymorphisms result in a diverse array of GR proteins (Oakley and Cidlowski, 2011). In recent years, it has become apparent that the plethora of GR isoforms have different functional properties when compared to the wild-type GR, termed GRα, and can influence GRα function or act independently (Oakley and Cidlowski, 2011). For example, in the human GR there is an acceptor splice site between exon 8 and 9 that results in a splice variant termed GRβ. GRα and β share the first 727 amino acids, thereafter the GRα possesses a further 50 amino acids and GRβ a non-homologous additional 15 amino acids. GRβ lacks the C-terminal helix 12 and the AF2 region, and so is unable to bind cortisol and induce transactivation. It does, however, form heterodimers with GRα to act as a dominant-negative inhibitor of GRα gene expression (Bamberger et al 1995), and has also been shown to directly stimulate or repress a number of genes not regulated by GRα (Kino et al 2009, Lewis-Tuffin et al 2007). A similar variant is present in zebrafish (*Danio rerio*), but the acceptor splice site is absent in exon 8 of other fish species and thus the presence of GRβ may be restricted to the Ostariophysi superorder in the fishes (Schaaf et al 2008). Zebrafish GRβ acts in a similar way as its vertebrate paralogue as a dominant-negative inhibitor of GRα, but similarly has recently been shown to regulate its own suite of genes (Chatzopoulou et al 2015). Another splice variant of the human GRα sees three bases retained from the intron separating exon 3 and 4, which

results in an additional amino acid, arginine, inserted between the two zinc fingers of the DBD this has been termed GRγ (Ray et al 1996, Rivers et al 1999). GRγ binds GCs with a similar affinity as GRα, but has an impaired ability to regulated GR transcription (Ray et al 1996), despite this, in various tissues GRγ can regulate a different subset of genes to GRα (Oakley and Cidlowski 2011) and has been associated with GC resistance in a number of cancers (Beger et al 2003). The alternate translation start sites in exon 2 have results in 8 versions of GRα (Oakley and Cidlowski, 2011). One of these, GRα-D, lacks the A/B domain, but is still active, regulating around 1800 genes in response to GCs (Oakley and Cidlowski, 2011). Furthermore, single nucleotide polymorphisms in GRα can also significantly alter function. Such an example is in GRα ER22/23EK polymorphism within exon 2, where a G to A point mutation in codon 22 results in a change of arginine to lysine; in patients with this mutation there is a concurrent silent G to A point mutation in codon 23 (van Rossum et al 2002). The result is a receptor with decreased hormone sensitivity, which is associated with patients with altered metabolism, risk of cardiovascular disease (DeRijk and de Kloet , 2005) and who are susceptible to depression (Panek et al 2014). The list of GR isoforms demonstrates that a wide range of regulatory strategies, including difference protein-ligand, protein-protein and protein-DNA interactions that mediate cellular specific glucocorticoid actions.

3. Vertebrate steroid receptor evolution

3.1 Hypothetical models for gene retention following duplication

Whole genome duplication (WGD) events play an important role in the evolution of complex organisms. An example would be the teleosts where a WGD event occurred between 320-350MYA (Hoegg et al 2004) which has resulted in the most specious vertebrate group containing around half of all known vertebrates (Glauser and Neuhaus, 2014). A WGD is undoubtedly a dramatic event and a large proportion (80-99%) of duplicated genes are lost (Jaillon et al 2004; Kassahn et al 2009, Woods et al 2005) and hypothetical models have

been developed to help explain why certain duplicated genes are retained (Ohno, 1970). In the duplication-degeneration-complementation model, (Force et al 1999) mutation in the encoding region of the duplicated gene may render the gene non-functional leading to its eventual loss from the gene pool. If duplicated genes are retained then they may either partially lose their ancestral function, thus, both are required to maintain full functional activity, a process known as sub-functionalisation, or neo-functionalisation may emerge where one gene retains the original function and the other alters to acquire a new function. A further model to explain retention of genes via sub-functionalisation termed escape from adaptive conflict (DesMarias and Ruahser, 2008) sees adaptive evolution driving changes in the two genes. Thus, in this scenario both paralogues are released from the potential negative effect of each other and both evolve to improve the subfunctional properties they carry out. Following a WGD the relative ratio of genes in a pathway is not altered. However, a disparity in this ratio will occur once mutations in one paralogue lead to altered function. Gene dosage, or maintenance of gene ratios, is thus an alternative mechanism by which duplicate genes may be retained and is hypothetically important for genes encoding proteins that function in gene pathways or networks, such as nuclear steroid receptors (Conant and Wolfe et al 2007).

3.2. Chordate and early vertebrate steroid receptors

The ancestral steroid hormone receptor (SR) is proposed to be “estrogen-like” and orthologs of ER genes are present in a number of invertebrate including the gastrapod *Aplysia californica* (Thornton et al 2003) and octapod *Octopus vulgaris* (Keay et al 2006), however, both ERs are constitutively active and do not respond to estrogens. In the cephalochordate, *Branchiostoma floridae*, there are two hormone receptor orthologs termed a steroid receptor (bfSR) and estrogen receptor (bfER) (Bridgham et al. 2008). The hormone transactivational properties of these two receptors have been characterised throught the method of cloning into a mammalian expression vector and assessing the activity of the recombinant proteins following transfection into mammalian cells along with reporter plasmid

containing either vertebrate estrogen response elements (ERE) or GRE upstream of the luciferase gene. Estrogen stimulated bfSR transactivation in the presence of the ERE containing plasmid, but not the GRE plasmid, where as GCs did not. In contrast, the bfER was transcriptionally unresponsive. However, the bfER acted as a negative regulator of bfSR, (Bridgham et al 2008) and would appear to play an analogous role to GRβ, which acts as a dominant-inhibitor of GRα gene activity in humans and zebrafish (Schaaf et al 2008).

Extant members of the earliest vertebrates, the agnathans (the hagfish and lamprey), possess 3 steroid hormone receptors that are homologous to the estrogen (ER), progesterone (PR) and corticosteroid receptors (CR) of other vertebrates (Bridgham et al 2006). The ER, PR and CR emerged following the duplication of cephalochordate SR and ER as a consequence of a WGD event early in the vertebrate lineage (Kuraku et al 2009). In transactivation studies the CR of the lamprey (*Petromyzon marinus*) and hagfish (*Myxine glutinosa*) is functional being activated by various corticosteroids such as cortisol, corticosterone, 11-deoxycortisol, 11-deocycorticosteorne, aldosterone and to a lesser extent by progestins, but not the androgens or estrogens (Bridgham et al 2006). In lamprey, 11-deoxycortisol has been shown to be an active glucocorticoid, elevating plasma glucose, and a mineralocorticoid, aiding ionoregulation (Close et al 2010). Computational analysis of the ligand binding pocket of lamprey CR by Baker et al (2011) identified that leucine-220 and methionine-299 made significant interactions between hydroxyl group on C17 of 11-deoxycortisol and the receptor (Close et al 2010), supporting the hypothesis that this steroid is the active corticosteroid in these ancient vertebrates.

The phylogenetic relationship between the lamprey and hagfish is debated (Bury et al 2016, Thomson et al 2014). Original classification suggested paraphyly with the lamprey being more closely related to the jawed fishes. However, recent molecular evidence suggest monophyly, with lampreys and hagfish forming a sister clade (Heimberg et al 2010), however, this has also been questioned (see Bury et al 2016 for further details). If these two groups are monophyletic it would suggest that the ancestral vertebrate was common to both,

as well as the gnathostomes. In this scenario it would appear that the lamprey has retained many of the ancestral features that also are present in extant gnathostomes, where as the hagfish has undergone a remarkable loss of these ancestral traits. For example, in the lamprey there is fully functional pituitary-interrenal axis, similar to that seen in the teleosts, which synthesises corticosteroid (Takahashi et al 2013) and produces an increase in circulatory concentrations in response to a stressor. A physiological role has also been identified (Close et al 2010). However, even though the hagfish possess a gene encoding for a CR (Bridgham et al 2006), the site of synthesis of corticosteroids has yet to be properly identified (Idler and Burton, 1976). Elevated circulating levels of corticosteroids equivalent to those measured in other vertebrates have seldom been recorded (Weisbart and Idler, 1970) and a physiological role of corticosteroids has not been identified (Bury et al 2016). Very few studies have tried to identify a role of the corticosteroids in the Myxini. Hagfish, in contrast to all other vertebrates, are osmoconformers in terms of Na^+ and Cl^-, but do regulate divalent ions and sulphate (Belamy and Jones, 1961). Interperitional administration of known corticosteroids (cortisol, corticosterone or 11-deoxycorticosterone) had little effect on their ability to deal with a sulphate challenge if a sulphate challenge was administered then no changes in plasma 11-deoxycortisol, the active corticosteroid in lamprey, was observed (Clifford et al unpublished results). Similarly, a stress protocol induced a rise in plasma glucose, but had no effect on plasma 11-deoxycortisol (Clifford et al unpublished results) and only after 7 days following administration of 11-deoxycorticosterone was there a minor increase in plasma glucose observed (Bury et al 2016). Consequently, an active corticosteroid system has yet to be clearly identified and characterised in Myxini.

3.3. Sarcoptygerian glucocorticoid and mineralocorticoid receptors.

There have been two further whole-genome duplication (WGD) events in the vertebrate lineage, one early on in the split from the chordates (Kuraku et al 2009) and the other in the Teleostei lineage (Hoegg et al 2004). The first of these WGD events resulted in duplicated CRs from which emerged the mineralocorticoid (MR) and glucocorticoid (GR) receptors

present in all extant vertebrates. The profile of steroid induced transactivation of the agnathan CR is similar to that of vertebrate MRs and would suggest that the ancestral CR was "MR-like" (Bridgham et al 2006). Sequence alignment and Maximum-Likelihood phylogenetic analysis of all known CRs has allowed for the prediction of the ancestral CRs at important nodes in vertebrate evolution (Bridgham et al 2006; Bridgham et al 2009; Carroll et al 2011). By characterising these predicted ancestral proteins and introducing site-directed mutations to engineer the molecules' evolutionary trajectories it has been possible to identify the permissive sequence of amino acid mutations in the ligand binding pocket region that conferred GR preferentially binding cortisol or corticosterone over aldosterone in the Osteichthyes (Bridgham et al 2006, Bridgham et al 2009, Harms and Thornton, 2014, Ortlund et al 2007) and the binding to 1β-hydroxycorticosterone in Chondrichthyes (Carroll et al 2008). The divergence in vertebrate GR and MR hormone selectivity is a potential way in which neofunctionalisation emerged. However, for the MRs mineralocorticoid specific action to evolve a further sequences of events was required. There are two 11β-HSD enzyme which catalyse the reaction that converts cortisol to cortisone: 11β-HSD Type 1 catalyses a reversible reaction, whereas 11β-HSD type 2 catalyses an irreversible reaction which reduces cellular cortisol concentrations by converting this hormone to the inactive cortisone. In mineralocorticoid responsive tissue such as epithelium involved in ionoregulation the MR and 11β-HSD type 2 are co-expressed to allow for the specific action of aldosterone (Whorwood et al 1994).

3.4. The ray-finned fish (Actinopterygii) glucocorticoid receptors

The genome of the spotted gar (*Lepisosteus oculatus*) possesses 1 GR, and a GR has also been cloned other basal ray-finned fish, a tropical gar (*Atractosteus tropicus*) (Oka et al 2014) and a sterlet (*Acipenser ruthenus*) (Li et al 2012). The sterlet GR shows a 9 amino acid insert between the zinc fingers of the DBD and this is encoded on exon 4 of the spotted gar gene (ENSLOCT00000012909). This insert is not present in the hagfish or lamprey CR (Bridgham et al 2006), Chondrichtyes GR (Carroll et al 2008) or tetrapod GRs (Hollenberg et

al 1985). Li et al (2012) and Oka et al (2015) reported only a single transcript of GR in the sterlet and tropical gar, respectively. However, ENSEMBL predicts a splice variant for the spotted gar GR that lacks this insert, thus there may be 2 GR variants in these ancient ray-finned fishes.

The WGD in the teleost lineage 350MYA (Jaillon et al 2004) resulted in further duplications of the GR and MR receptors. Extant teleosts appear to have lost one of the MR duplicates as only one MR is present in those fish whose genomes have been sequenced. Li et al (2012) identified two GRs in an extant member of the basal teleost the Osteoglossimorph, *Pantadon buchholzi* – an order that split from the acipensideridae and was derived following the teleost WGD (Hoegg et al 2004). They identified two isoforms of GR, one termed GR1 containing the 9 amino acid insert between the DBD, previously observed in the basal ray finned fish, and one without termed GR2, in addition a splice variant of GR1 lacking the 9 amino acids was also identified (Li et al 2012). Whole genome sequencing of teleosts and further cloning of full-length fish GRs has revealed that the majority of fish have retained duplicated GRs (Figure 1; Supplementary Material Table and Figure; Bury et al 2003, Greenwood et al 2003, Kim et al 2011, Miyagawa et al 2014, Stolte et al 2007,). The GRs split into two groups; those possessing this 9 amino acids between the zinc fingers of the DBD, GR1, and those that do not, GR2 (Figure 1). Similar to the spotted gar these amino acids are encoded on a separate exon [e.g. exon 5, Stickleback (*Gasterosteus aculeatus*) GR1 (ENSGACT00000027452); exon 3, Fugu (*Takifugu rubripes*) (ENSTRUT00000015714); exon 3 Platyfish (*Xiphophorus maculates*) (ENSXMAT00000001516); exon 3 cavefish (*Astyanax mexicanus*) (ENSAMXT00000020636); exon 3, Amazon molly (*Poecilia formosa*) (ENSPFOT00000005871), exon 4 Nile tilapia (*Oreochromis niolticus*) (ENSONIT00000010671); exon 19 Cod (*Gadus morhua*) (ENSGMOT00000019605)]. There are reports of splice variant of the teleost GR1 without these amino acids, thus there are 3 GRs present in teleost fish, a GR1, termed GR1a, and a splice variant of GR1, termed Gr1b

and GR2 (Greenwood et al 2003, Li et al 2012, Miyagawa et al 2014, Stolte et al 2008, Takeo et al 1996). The number of genes retained following duplication is disputed, but is estimated to be between 1 and 20% (Aparico et al 2002, Kassahn et al 2009, Woods et al 2005), thus, an assumption would be that the retention of 2 GRs in teleosts following the WGD event 350 MYA (Hoegg et al 2004) offered an evolutionary advantage.

The exception to this is the zebrafish which have one gene encoding a GR, which lacks the 9 amino acid insert, and groups with the teleost GR2 (Schaaf et al 2008). It is not known if the loss of a GR is seen in other fishes or restricted to the zebrafishes. The GRs of only 3 species of the Ostariophysii, to which the zebrafish belong, have been cloned to date and all are in the cyprinidae. Filby and Tyler (2007) report the cloning of 1 GR in fathead minnow, however, in contrast Stotle et al (2007) reports duplicated GRs in the common carp. A further common carp WGD event occurred relatively recently 8MYA (Li et al 2015), which may account for the duplicated GR. However, analysis of 1757 recently duplicated common carp genes identified by Li et al (2015) shows that the paralogues amino acid sequences are 90% similar. By contrast there is only 57% similarity between the amino acids of carp GRs (Stolte et al 2007). In addition, the common carp GR1 possess the extra 9 amino acid insert characteristic of this teleost GR group (Stolte et al 2007) and the other GR groups with the other teleost GR2s (Figure 1). Consequently, the most parsimonious conclusion is that the two GRs in common carp are not from this recent carp lineage WGD and that a loss of the GR only occurs in the lineage of fishes that includes the zebrafish following the split from the common carp.

3.4.1. Teleost glucocorticoid receptor DNA binding domain

The significance of this actinopterygian lineage specific 9 amino acid insert in the DBD of GR has been puzzling since its discovery in the rainbow trout GR1 by Ducouret et al (1995). The 9 amino acids generate an additional loop that resides outside of the protein (Wickert and Selbig, 2002) and transactivational activity indicates that this extra loop does not affect

recognition of consensus GREs (Ducouret et al 1995). Functional studies on the transactivational properties of the GR1a and GR1b splice variants do show some differences in activity, but this is species specific and depends on the structure of cis-regulatory region. Takeo et al (1996) showed that both rainbow trout GR1 splice variants were active in the presence of cortisol and dexamethasone using a reporter plasmid containing the full-length mouse mammary tumor virus (MMTV) long terminal repeat sequence. Using a different reporter plasmid, but one that also contains the MMTV sequence, Miyagawa et al (2014) found that the transactivational activity EC50 for the Japanese medaka GR1 splice variants increased and there was a decrease in fold induction (Miyagawa et la 2014). The presence of the insert in *Haplochromis burtonii* GR1 results in a reduction in the maximal transcriptional activity in a system using a reporter plasmid containing 3 GREs (Greenwood et al 2003), and in the rainbow trout differences between the splice variants emerge, with reporter plasmids possessing fewer GREs. Lethimonier et al (2012) showed that the splice variant lacking the insert is unable to interact with a single GRE, but function is restored with the reporter plasmid contains two GREs; with the full length GR1 activates both (Lethimonier et al 2002).

There are species within the GR2 group that also possess additional amino acids insertion in the DBD. For example, the first study to clone a second GR in a teleost fish found that the rainbow trout GR2 has an insertion of 5 amino acids, GTGAR, in this region (Bury et al 2003). Subsequent sequencing identified a similar insertion in another salmonid, Atlantic Salmon (Supplementary Material Table). The salmonid lineage has experienced a WGD event approximately 50 – 80 MYA (MacQueen and Johnson, 2014), but this 5 amino acid insertion is not a consequence of this event and is present in other Protacanthopytergii, such as the Esociform, the European Pike (*Esox lucius*) (Supplementary Material Table). Kim et al (2011) generated a marine medaka GR2 mutant that contained the addition of the 9 amino acids present in GR1 and assessed transactivational activity and ability of the receptors to bind transcription co-regulators, GRIP 1, a co-activator (Avenant et al 2010), and SMILE a

co-repressor (Xie et al 2009). The co-transfection of each receptor with plasmids expressing mouse GRIP enhanced activity, whereas mammalian SMILE repressed activity, as expected, however, the GR2 mutant containing the 9 amino acids showed increased transcriptional activity compared to GR2 wild type, demonstrating the significance of the insert in regulating transcriptional activity.

The significance of these variations in the GR DBD is currently not clear, however due to the fact they are present in basal actinopterygians and early teleosts, and have been retained in almost all other extant teleosts it is suggested that they offer an evolutionary advantage. Whether this has enabled the GR isoforms to recognise different response elements and be retained via subfunctionalisation or whether they have co-evolved with changes in the non-coding regulatory regions of genes to control different gene pathways of the ancestral vertebrate GR awaits further study. Interestingly, a recent study by Kiilerich et al (2015) identified that the rainbow trout MR, which is also activated by cortisol and in the absence of defined teleost mineralocorticoid or function for MR (Takahashi and Sakamoto, 2013) could be described as a third teleost GR, is a repressor of both rtGR1 and rtGR2 transcriptional activity (Figure 3). Similar observations have been made with the mammalian GR and MR and the repressor activity is due to GR/MR heterodimerisation that disrupts the self-synergistic interactions between the N-terminal of 2 GRs when bound as a homodimer (Liu et al 1995). However, point mutations in the DBD of the MR suggests that in trout the dominant-negative effect of rtMR on the rtGRs is associated with DNA recognition. This effect was more prominent in the rtGR2 and if plasmids containing 1 or 2 GREs were used (Kiilerich et al 2015).If GR1a and b, GR2, MR co-localise then there is the potential for 6 different heterodimers that could dampen or enhance the transcriptional activity of one or other of the receptors (Figure 3). Nuclear receptor heterodimer formations is a common mechanism of transcription regulation in other receptors (RXR, VDR, PPAR and TR (Gronemeyer et al 2004) and thus may also be an important regulatory mechanism to mediate the action of GCs via GR1 and GR2.

3.4.2. Teleost glucocorticoid receptor hormone sensitivity

The first GR2 to be cloned and characterised was from the Rainbow trout, and showed distinct differences in functionality between itself and rtGR1 - rtGR2 had a greater increase in hormone transcriptional activity at equimolar hormone concentrations and increased sensitivity (Bury et al 2003). The ability to repress NFκB transcriptional activity also occurred at lower dexamethasone concentrations with GR2 (Bury and Sturm 2007) and the movement of GR2 from the cytoplasm to the nucleus also occurred at lower hormone concentrations (Becker et al 2008). In this initial study (Bury et al 2003) a cell free expression system was used to assess receptor hormone binding and found no difference in affinity for dexamethasone between the two GRs. However, a subsequent study using a cell line expression system found rtGR2 to have an increased hormone binding affinity (Sturm et al 2011). This difference in sensitivity is not restricted to the salmoniformes (Table 1) and a similar transactivation activity pattern is seen with the two GRs from the basal teleost *P. buchholzi* (Li et al 2012), carp (Stolte et al 2007), marine medaka (*Oryzias dacena*) (Kim et al 2011), and the Japanese medaka (*Oryzias latipes*) GRs show a remarkable 10 000 fold difference in the cortisol EC50 for transactivational activity (Miyagawa et al 2014). Focusing on the rainbow trout GRs, two papers by Sturm et al (2010, 2011) aimed to identify the molecular signatures that conferred the difference in the rtGR transcriptional sensitivity to glucocorticoids. Deletion of the NTD reduced transactivational activity to 2% of the wild type (Sturm et al 2010), as is seen with mammalian GRs (Giguere et al 1986), but did not alter the sensitivity, suggesting that the HBD plays a significant role. This was confirmed with chimeric constructs where by the HBD of the two receptors where exchanged and the chimera that contained the HBD of either GR1 or GR2 resembled the sensitivity of the respective wild type (Sturm et al 2011). When 3 sub-regions of the HBD were exchanged it was apparent that each contributed to the differences in sensitivity. However, the C-terminal extremity of GR1 differs to GR2, with Ala and Leu (AL) replacing the consensus C-terminus sequence GluLys (QK) (Supplementary Material Figure) and also containing an additional 6

amino acids. When these 6 amino acids are deleted and AL converted to QK the mutant GR1 increases its hypersensitivity by 4.1 fold (Sturm et al 2011). Using the crystal structure of the dexamethasone bound GR hormone binding domain Bledsoe et al (2002) indentified that a β strand situated after the AF2 located in helix 11 and 12 at the C-terminal region interacted with a β-strand located between Helix 8 and 9 in the HBD to stabilise the active AF2 configuration. Thus, these additional amino acids of rtGR1 may have the potential to affect the stability of the active receptor. Other protacanthopytergian GR1, including the salmonids, Atlantic salmon (*Salmo salar*) and Brown trout (*Salmo trutta*), the coregonid, Marena whitefish (C*oregonus maraena*) and escoid, European pike (*Esox lucius*) also possess additional amino acids at the C-terminus (see Supplementary Material Figure). Interestingly, the hyposensitive GR1 of *P. buchholzi* possesses the conserved QK, but an additional 21 amino acids. However, there is always an exception and the Japanese medaka GR1 possess no additional amino acids in this region but do have the amino acids SS at the N-terminus as opposed to the consensus QK (Supplementary Material Table).

These differences led to a hypothesis that the two teleost GRs had been retained due to a difference in their hormone sensitivity, with the hypersensitive GR playing a more prominent role in regulating gene pathways during periods of basal circulatory concentrations of hormone (unstressed), with the hyposensitive GR becoming more prominent during stressful stimuli when plasma hormone concentrations are elevated (Bury et al 2003). The observation of hypo and hyper-sensitive GRs in an extant member of one of the first teleost groups to emerge following the teleost lineage WGD would support this hypothesise. However, Greenwood et al (2003) reports no significant difference in EC50 for cortisol for the two *Haplochromis burtonii* GRs (Table 1).

4. Conclusion

The teleost WGD event resulted in the duplication of the vertebrate GR and MR and two GRs have been retained in the majority of teleost fish studied so far. The molecular basis for

this retention has yet to be fully understood. The cloning of the first GR2 and functional characteristic analysis of subsequent GR2 suggested that there is one mechanism for differential regulation of gene networks maybe via differences in the transcriptional activity sensitivity of the 2 GRs, (Bury et al 2003). This maybe the case in the rainbow trout (Bury et al 2003), carp (Stolte et al 2007) and beloniforms (Kim et al 2011, Miyagawa et al 2014), but was not observed in a cichlidae (Greenwood et al 2003) and thus is not a universal explanation for the retention of the two GRs. The only common feature is the 9 amino acid insert in the teleost GR1 group. Very little work has been carried out to assess the significance of this insertion, but those studies that have suggest that the insertion does indeed affect transcriptional responses, however, this is dependent on species and the number of GRE upstream of reporter genes (Lethiomonier et al 2002, Greenwood et al 2003, Miyagawa et al 2014). An interesting observation is the effect of heterodimer formation in regulation of receptor function. In the earliest active SR reported from a cephalochordate, its transcriptional activity is repressed in the presences of a non-active ER (Figure 3A., Bridgham et al 2008). The ability to repress GR activity also appears to be of importance in vertebrates. In humans, a splice variant GRβ, which lacks the C-terminal region, is a negative-dominant repressor of GRα (Figure 3B, Bamberger et al 1996). In zebrafish, which have lost the second GR found in other teleost fishes, convergent evolution sees the emergence of a similar GRβ to that in humans that also acts as a repressor of zebrafish GRα activity (Figure 3B, Schaaf et al 2008). To date, the zebrafish are the only fish species known to have “human-like” GRβ, but GR activity repression may occurs via a different route in other fish species, with Kiilerich et al (2015) showing that in rainbow trout the MR, which also binds cortisol and can activate GREs in vitro (Sturm et al 2005), can act to repress GR1 and GR2 transactivation

The retention of teleost GRs suggests some evolutionary advantage that saw the development of a more complex regulatory process to mediate the actions of glucocorticoids. This may be due to either difference between the GRs in their sensitivity to

hormones, their DBD recognising different GREs, or heterodimer formation altering the functional properties and in doing so altering the ability to activate genes. However, the regulation of tissue specific GC actions maybe even more complex, due to the fact that a number of vertebrate GR proteins have been identified that lack portions of the NTD due to different translation initiation sites and post-translational modifications will affect functionality (Oakley and Cidlowski, 2011).

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Figure 1. Phylogenetic tree using 75 full length actinoptergyian GRs and hagfish CR. Tree was constructed with the Maximum-Likelihood methods in MEGA6 (Tamura et al 2013) using the Jones-Taylor Thornton model and nearest-neighbour interchange. Bootstrap values are reported based on 800 replicates. The tree shows clearer the two GR1 and GR2 groups with GRs from orders of fish clustering. The hagfish CR is separate and the basal actinotperygian GRs (*Acipenser ruthenus*, *Lepisosteus oculatus* and *Atractoseus tropicus*) group together between the teleost GR1 and GR2.

Figure 2. The consensus amino acid sequence spanning the two zinc fingers of the teleost GR. A. Represents the 5 additional amino acid insert of GR2 observed in Salmoniformes and Escoiformes. B. Represents the 9 amino acid insert seen in the GR1 group (also see Supplementary Material Figure), splice variants of GR1 exist where these 9 amino acids are absent.

Figure 3. Evidence for repression of steroid receptor function due to heterodimer formation. A. The situation in the cephalochordate where a steroid receptor (SR) is transactivationally active (represented by a solid arrow) in the presence of estrogens in contrast the estrogen (ER) is inactive (represented by an arrow with a cross), but acts a repressor (represented by a dashed arrow) of SR activity. B. In humans and zebrafish a spice variant in exon 8 forms a truncated glucocorticoid receptor (GR), termed GRβ, that acts as a repressor of GRα activity. There is also evidence of MR/GR heterodimer formation repressing GR activity. C. In teleost fish, the situation is more complex, the mineralocorticoid receptor (MR) has been shown to be transcriptionally active, but represses the actions of GR1 and GR2. It is unclear (represented by a dashed arrow with a question mark) how the various isoforms of GR in fishes influenced each others, either via repression or enhancement, function.

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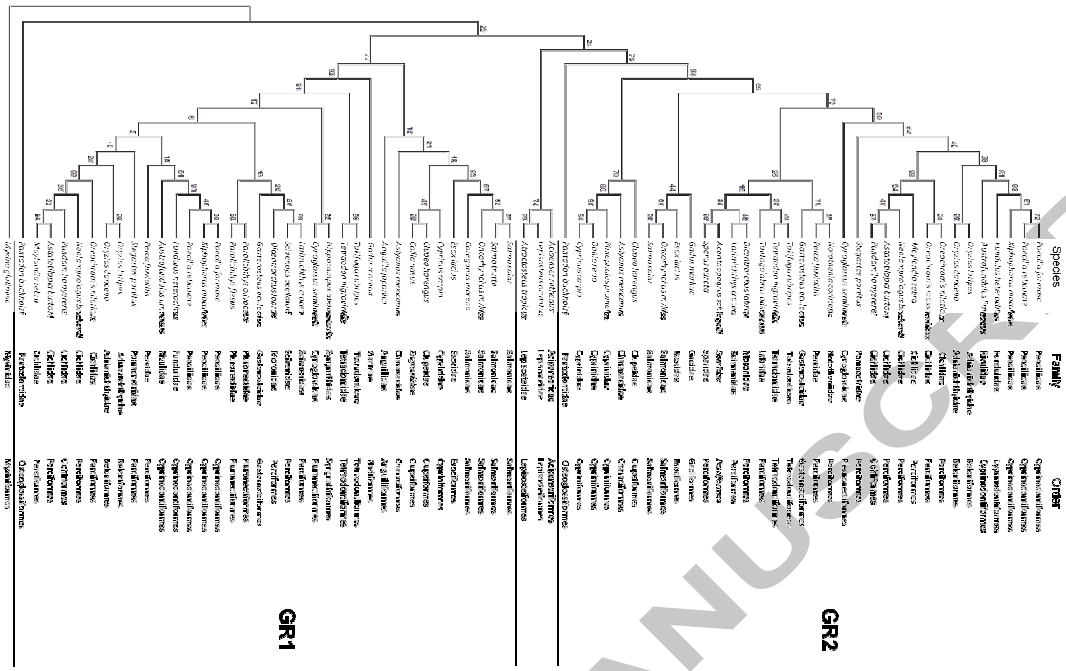

Figure 1

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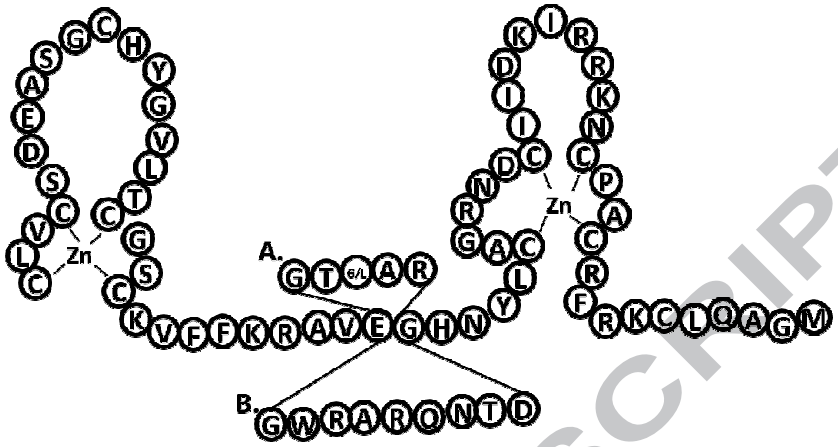

Figure 2

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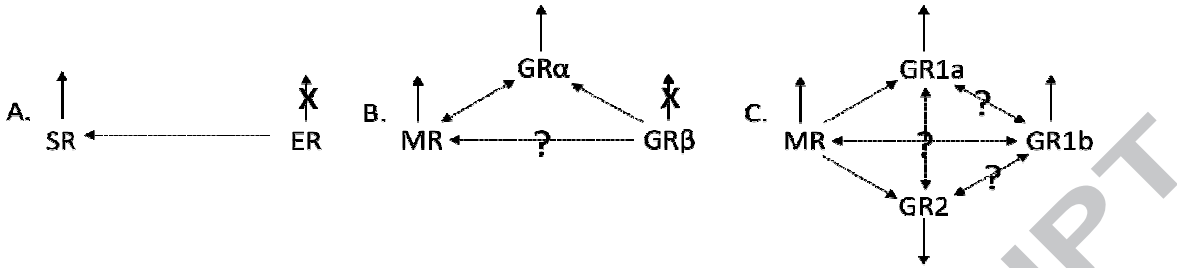

677 Figure 3

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Table 1 Transactivation EC50 values for full length actinopterygii GRs.

Species	EC50		Reference
	GR1	GR2	
Rainbow trout (*Oncorhynchus mykiss*)	46 ± 12nM[a] 4.4 nM[b]	0.72 ± 0.87nM[a] 0.6nM[b]	Bury et al (2003) Sturm et al 2011
Common Carp (*Cyprinus carpio*)	7.1 ± 2.9nM[a] 2.4 ± 3.8nM[b]	2.4 ± 0.4nM[a] 0.7 ± 1.4nM[b]	Stolte et al (2007)
Marine medaka (*Oryzias dancena*)	21.8 ±1.1nM[a]	9.9 ± 2.5nM[a]	Kim et al (2011)
Japanese medaka (O*ryzias latipes*)	57nM[a]	0.00085nM[a]	Miyagawa et al 2014
Haplochromis burtoni	5.4nM[a]	3.6nM[a]	Greenwood et al (2003)
Pantadon buchholzi	10.4 ±1.4nM[a,1] 12.0 ± 1.3nM[b,1]	2.7 ±0.6nM[a] 1.5 ± 0.4nM[b]	Li et al (2012)
	GR		
Zebrafish (*Danio rerio*)	10.1nM[a]; 0.37nM[b]		Schaaf et al 2008
Tropical gar (*Atractosterus tropicus*)	1.3nM[a], 0.15nM[b]		Oka et al (2015)
Sterlet (*Acipenser ruthenus*)	21.6 ± 3.1nM[a], 2.2 ± 0.7nM[b]		Li et al (2012)

a – cortisol, b – dexamethasone. 1 – EC50 for GR1b which lacks the 9amino acid insert.

679 **Highlights**

680

681 The majority of teleost fish possess 2 glucocorticoid receptors termed GR1 and GR2

682 GR1 possess a 9 amino acids (aa) inserts between the zinc fingers of the DNA binding
683 domain

684 The 9 aas are absent in teleost GR2, as well as tetrapod and Chondrichtyes GRs

685 The 9 aa insert is unique to the Actinopterygii being also present in basal ray-finned fish.

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