REVIEW

Glucocorticoid receptor activities in the zebrafish model: a review

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Abstract

Glucocorticoids (GCs) are steroid hormones that contribute to the regulation of many physiological processes, such as inflammation, metabolism and stress response, mainly through binding to their cognate receptor, GR, which works as a ligand-activated transcription factor. Due to their pleiotropy and the common medical use of these steroids to treat patients affected by different pathologies, the investigation of their mechanisms of action is extremely important in biology and clinical research. The evolutionary conservation of GC physiological function, biosynthesis pathways, as well as the sequence and structure of the GC nuclear receptors has stimulated, in the last 20 years, the use of zebrafish (a teleost of Cyprinidae family) as a reliable model organism to investigate this topic. In this review, we wanted to collect many of the most significant findings obtained by the scientific community using zebrafish to study GCs and their receptors. The paper begins by describing the experiments with transient knockdown of zebrafish *gr* to gain insights, mainly during development, and continues with the discoveries provided by the generation of transgenic reporter lines. Finally, we discuss how the generation of mutant lines for either *gr* or the enzymes involved in GC synthesis has significantly advanced our knowledge on GC biology.

Key Words

- ▶ glucocorticoid receptor
- ▶ glucocorticoids
- ▶ signaling pathways
- ▶ transcriptional regulation
- ▶ *Danio rerio*
- ▶ zebrafish
- ▶ morpholino knockdown
- ▶ transgenic reporter lines
- ▶ mutant lines

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The glucocorticoid signaling pathways

Steroid hormones are lipophilic and low-molecular weight compounds broadly involved in endocrine signaling, thus, able to trigger systemic effects through bloodstream circulation. All steroid hormones derive from cholesterol, whose basic structure is cyclopentanoperhydrophenanthrene, a molecule characterized by a core containing 17 carbon atoms fused to form four rings. Specific functional groups attached to these rings determine the precise chemical properties of the different steroids.

These hormones can be grouped into two main classes: corticosteroids and sex steroids, according to their major

site of synthesis and function. Particularly, corticosteroids include glucocorticoids (GCs) (corticosterone, 11-dehydrocorticosterone, cortisol, and cortisone) and mineralocorticoids (aldosterone, 11-deoxycorticosterone), while sex steroids include androgens (androstenedione, testosterone, 5 α -dihydrotestosterone), estrogens (estrone, estradiol), and progestogens (progesterone).

In humans, biosynthesis and release of GCs predominantly take place in adrenal cortex where it is regulated by the hypothalamus–pituitary–adrenal (HPA) or hypothalamus–pituitary–interrenal, HPI, in fish) neuroendocrine axis. Moreover, local GC synthesis also

occurs in different tissues, including lymphoid organs, skin, brain, intestine and cardiovascular system (Taves *et al.* 2011).

GCs mainly exert their systemic functions through the binding to the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). Due to their steroid lipophilic nature, GCs can cross cell membranes and bind their intracellular receptors. GR resides in the cytoplasm in almost all mammalian tissues and is involved in a wide spectrum of different systemic effects, such as growth, reproduction, bone formation, vascular tone, brain functions and immune response (Nicolaidis *et al.* 2010). In humans, GR is translated from a single gene, termed *NR3C1*, while different protein isoforms are generated by various mechanisms, such as alternative splicing, alternative translation initiation, and posttranslational modifications (ubiquitination, SUMOylation, phosphorylation, acetylation) (Oakley & Cidlowski 2013). In more detail, the *hNR3C1* gene is located on chromosome 5 and consists of nine exons. The alternative splicing of exon 9 results in the production of two different mRNAs coding for either the α or β isoform. At their C-terminal, after a common sequence of 727 amino acids (aa), the α and β isoforms display a stretch of 50 and 15 non-homologous aa, respectively. While hGR α is a cytosolic protein having the classical functions of a GC cognate receptor, the hGR β roles are not completely understood: the protein is unable to bind GCs and is thought to exert its activity as a dominant-negative inhibitor of GR α (Nicolaidis *et al.* 2010, Quax *et al.* 2013). Additionally, GR α -independent, gene-specific transcriptional activities have been described for GR β , at least in *in vitro* models (Lewis-Tuffin *et al.* 2007, Kino *et al.* 2009).

Evolutionary analysis revealed that all members of the steroid receptor family derive from a single ancestral estrogen receptor (ER) and share the same modular structure (Eick & Thornton 2011).

Accordingly, the N-terminal portion of GR contains a transactivation domain (NTD) followed by the two zinc-finger motifs of the DNA binding domain (DBD). The carboxy-terminal domain, that is separated by a hinge region from the DBD, contains the GC binding site (LBD) and a second region with activation functions (AF2) (Kadmiel & Cidlowski 2013). In the absence of ligands, inactive GR is stuck in the cytoplasm, forming a multimeric complex with other proteins, including heat shock protein (HSP) 90, HSP70 and immunophilins, and is unable to promote any genomic effect. Upon binding with the ligand, GR was assumed to dissociate from the repressor

protein complex and migrate to the nucleus to regulate gene transcription. This classical view of GC action has recently been revised and described as a more complicated process in which, after binding with GCs, the composition of the multimeric complex changes: immunophilin FKBP51 (FK506-binding protein 51), a co-chaperone protein that binds HSP90 and decreases the affinity of GR for cortisol, is replaced by FKBP52 (FK506-binding protein 52), recruiting dynein to support translocation of the GC/GR complex to the nucleus (Binder 2009). Finally, the heterocomplex between GR and HSP90, enter through the nuclear pore complex and dissociates when inside the nucleus (reviewed by Daneri-Becerra *et al.* 2019) (Fig. 1).

Once into the nucleus, either in a monomeric or dimeric form, the GC/GR complex is able to modulate gene expression in different ways: either by direct binding to DNA, or by tethering to other DNA-bound transcription factors, or in a composite manner that requires direct interaction with DNA and adjacent DNA-bound transcription factors (Oakley & Cidlowski 2013). The overall effect of these interactions may result in the stimulation or repression of gene transcription (Kadmiel & Cidlowski 2013) (Fig. 1). GR is able to bind glucocorticoid response elements (GREs) consisting of palindromic 5'-GGTACAnnnTGTTCT-3' DNA sequences, in which 'n' could be any nucleotide (Beato & Klug 2000). Remarkably, the response elements of other steroid receptors that share with GR the same common ancestor and conformational similarities, such as androgen receptor (AR), progesterin receptor (PR), and mineralocorticoid receptor (MR), show strong resemblance, despite their different genetic, genomic and physiological functions (Beato & Klug 2000, Geserick *et al.* 2005). Because of this promiscuity, some authors refer to GREs as hormone responsive elements (HREs) (Beato & Klug 2000). The specificity of the response relies on several factors like cell-specific expression (this is not always the case), tethering with other transcriptional regulatory elements (co-factors) as well as co-activators (Geserick *et al.* 2005, van Weert *et al.* 2017), variation of the DNA binding sequence as well as specific contact of the receptors with the flanking regions (reviewed in Veras Ribeiro Filho *et al.* 2019). This allows that, despite strong similarities in their responsive elements, co-expression and activity of different receptors in the same cell can result, in combination with each other and with other transcription factors, in the transcriptional regulation of different genes (Severson *et al.* 2018).

Pieces of evidence also suggest that GCs can exert some of their functions by non-genomic rapid response mediated by either membrane-bound GR

Figure 1

Glucocorticoid genomic and non-genomic signaling. GCs are small lipophilic molecules that are able to cross membranes and trigger a plethora of different and partially unrevealed molecular responses. GCs may bind cytoplasmic or membrane GR to exert their rapid, non-genomic biological function interacting with other proteins or shifting their localization to the mitochondria. Mechanisms of enzymatic cytosolic inactivation or reactivation of GCs operated by 11 β -hydroxysteroid dehydrogenase type 1 and type 2, may regulate GC viability. In the absence of ligands, inactive GR is located in the cytoplasm, forming an inactive multimeric complex with other proteins. Upon binding with the ligand, the immunophilin FKBP51 is replaced by FKBP52, which recruits dynein motors and allows GC/GR translocation to the nucleus where GR dissociates from HSP90. The genomic response may act through at least three different mechanisms: direct binding in which GC/GR can bind DNA elements, functioning as positive (GRE) or negative (nGRE) regulation elements; tethering in which GC/GR may physically interact with other transcription factors to regulate gene expression and composite binding in which GC/GR requires both GRE elements and physical interaction with other transcription factors to exert its transcriptional activity. BTM: basal transcription machinery.

(Groeneweg *et al.* 2012, Scheschowitsch *et al.* 2017) or direct interactions with different kinases, such as protein kinase B (PKB), phosphoinositide 3-kinase (PI3K), and mitogen-activated protein kinases (MAPKs) (Oakley & Cidlowski 2013). Additionally, after binding to GCs, GR α can translocate into mitochondria and regulate mitochondrial transcription (Lapp *et al.* 2019) (Fig. 1).

In mammals, as expected by the almost ubiquitous expression of their receptor, GCs regulate many physiological processes, including metabolic homeostasis, immune system, reproduction, behavior and stress response (Whirledge & DeFranco 2018).

The employment of GR mutant mouse (GR $^{-/-}$), obtained by homologous recombination, demonstrated that GCs are required for correct development and organogenesis (Cole *et al.* 1995). This mutant line was

crucial to determine how liver, adrenal gland, brain, and HPA axis are affected by GR knockout during development (Cole *et al.* 1995, Schmid *et al.* 1995). On the other hand, GR $^{-/-}$ mice die within a few hours after birth, due to lung insufficiency and respiratory failure; this represents a major limitation of the KO mouse model (Cole *et al.* 1995). Taken together, these results suggest that GC signaling pathways, while playing a pivotal role in postnatal life, are also essential during development. However, the KO mouse model is not informative enough to satisfactorily elucidate the role of GR functions in the early phases of embryonic development, mostly because of the inaccessibility to the mouse embryo. Yet, it is not possible to explore the signaling pathway in adulthood either, because of the premature death for lung insufficiency.

Therefore, conditional and inducible KO mice were produced to study the relevance of GC signaling pathways in a physiological context (recently reviewed by Whirlledge & DeFranco 2018). For example, GR was specifically deleted in the brain, revealing that the absence of this signaling in the CNS leads to reduced anxiety-related behavior, thus highlighting the role of GCs as a behavioral modulator (Tronche *et al.* 1999). As reported by Whirlledge & DeFranco (2018), conditional mice were generated to characterize specific functions of GR in numerous cell types, sometimes with contradictory data, possibly due to different experimental approaches and genetic background of mouse strains. These models are extremely important for the functional analysis of GR signaling in specific tissues and cell types, although the possibility to analyze GR null models is desirable since alteration in GC concentration leads to responses at the global level of an organism.

GC imbalance is associated with pathological conditions. GC chronic increase or decrease can result in complex endocrine disorders involving the adrenal cortex and are known as Cushing's and Addison's disease, respectively (Kadmiel & Cidlowski 2013). An excess of cortisol, along with bone loss, metabolic diseases and high blood pressure, is linked with distinctive changes in physical appearance; patients who are affected by Cushing's disease are more likely to develop cardiovascular diseases (Fardet *et al.* 2012). On the other hand, since Addison's disease is due to lack of cortisol and aldosterone production by the adrenal gland, people affected by this disease are characterized by low blood pressure, hyperpigmentation and muscle weakness (Napier & Pearce 2014). Moreover, abnormally high levels of GCs are found to be associated with psychiatric diseases, such as schizophrenia, mood disorders and post-traumatic stress disorders (Silverman & Sternberg 2012), as well as drug and alcohol dependence (Ambroggi *et al.* 2009).

Interestingly, synthetic GCs, being able to inhibit pro-inflammatory transcription factor like nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) and promoting anti-inflammatory genes such as the interleukin 10 (IL-10), are widely administered as therapeutic compounds to contrast inflammation and other symptoms of autoimmune disorders or lymphoproliferative diseases (Yasir *et al.* 2020).

Cortisone was first administered for therapeutic purposes in the late '40s by Philip Hench. The physician, who used it to treat the inflammation due to rheumatoid arthritis, was later awarded the Nobel prize for Physiology or Medicine in 1950 (Burns 2016).

On the other hand, GC-based therapies may carry some adverse effects that should not be neglected: premature arteriosclerosis, arrhythmias, gastric ulcers, glaucomas, cushingoid features (Yasir *et al.* 2020).

The study of GC/GR signaling in *Danio rerio*

The wide implications of GCs in physiological and pathological conditions make their signaling a fascinating field of scientific research. The difficulties encountered in mammalian models, such as the limited number of offspring, the inaccessibility of the mouse embryos and the impossibility of raising *GR*^{-/-} mice to adulthood, can be overtaken by broadening the model species used to study these hormones.

Zebrafish (*D. rerio*), a freshwater fish, is largely employed as a model organism in scientific research for its intrinsic properties, such as abundance of offspring, external fertilization and development, remarkable optical accessibility of embryo at any developmental stage from fertilization. In addition, *D. rerio* shares extensive genetic, physiological and molecular similarities with mammals, features that make it an excellent model for developmental studies (Balasubramanian *et al.* 2019), drug discovery (MacRae & Peterson 2015), cancer biology (Lobert *et al.* 2016), ecotoxicology (Bambino & Chu 2017), as well as endocrinology and GC/Gr research (McGonnell & Fowkes 2006, Schaaf *et al.* 2009, Löhr & Hammerschmidt 2011).

Despite a high conservation between zebrafish and human of the main steroidogenic pathways, suggesting a general conservation of the molecular and cellular processes, some differences are evident, particularly concerning the synthesis of some teleost-specific steroids (Tokarz *et al.* 2015). The mineralocorticoid receptor (Mr) is present in teleosts but is activated by 11-deoxycorticosterone and cortisol (Pippal *et al.* 2011). Aldosterone, the main mammalian mineralocorticoid, is not synthesized in teleosts and is a synapomorphic trait of the tetrapods clade, needed to maintain ion balance after the transition from the water to the land (Colombo *et al.* 2006, Tokarz *et al.* 2015, Baker 2019). In addition, a notable difference between teleost and mammals is the anatomical location of the GC secreting cells. In most teleosts, including zebrafish, the interrenal cells involved in GC synthesis is interspersed throughout the head kidney. Hence, the axis that regulates GC synthesis is called HPI (Alsop & Vijayan 2009, Löhr & Hammerschmidt 2011).

Despite the genome duplication occurred in the teleost clade (Volf *et al.* 2005), zebrafish carries a single copy of the *gr* gene (*nr3c1*), located on chromosome 14, while the second copy has possibly been lost recently, likely as a consequence of chromosome 21 rearrangements (Schaaf *et al.* 2008). The human and zebrafish genes for GR show remarkable similarities in their structure, including an alternative splice variant processing leading, also in zebrafish, to a Gr β isoform (Schaaf *et al.* 2008). However, recent studies suggest that, at least in zebrafish, the Gr β isoform is not involved in transcriptional regulation (Chatzopoulou *et al.* 2017).

The aim of this review is to highlight how zebrafish models were employed to elucidate the molecular pathways underlying the GC-activated signaling and

show how the endocrinological field may further benefit from studies in *D. rerio*.

Morpholino-induced knockdown of glucocorticoid receptors in zebrafish

Zebrafish fertilization, being external, allows the microinjection of small antisense oligomers, called morpholinos (MOs), to transiently inhibit the function of target genes by hampering either translation (ATG-MO) or splicing (SPL-MO) of their mRNA. Since the early 2000s, this practice has been the most common to overcome the lack of efficient, targeted mutagenesis methods in zebrafish (Stainier *et al.* 2017) (Fig. 2).

Figure 2

Main citations and results on *gr* morpholino.

First experiments with MOs were performed with *gr* SPL-MO and revealed a negative role for GCs on zebrafish caudal fin regeneration after its amputation (Mathew *et al.* 2007). Later on, the same approach was used to identify compounds, including different synthetic GCs, able to modulate this process (Sengupta *et al.* 2012). More recently, further investigations demonstrated that a strong Gr-dependent upregulation of the oncofetal gene *cripto-1* is possibly involved in the GC/Gr-mediated inhibition of regeneration (Garland *et al.* 2019).

Notably, the knockdown approach with *gr* SPL-MO by Mathew *et al.* (2007) did not cause any overt developmental defect on embryos and larvae. This result was later confirmed by other papers and it is suggestive of a non-essential role of zygotic Gr during early zebrafish development (Pikulkaew *et al.* 2011).

Nonetheless, in teleosts, including zebrafish, *gr* mRNA is the most abundant among the maternal transcripts of steroid hormones receptors (Pikulkaew *et al.* 2010), together with maternally derived cortisol (Alsop & Vijayan 2008). The bioactivity of cortisol and the relevance of *gr* maternally supplied for normal development were demonstrated by the fact that ATG-MO knockdown of maternal transcripts produces multiple developmental defects including compromised embryonic and larval survival, morphological alterations, abnormal mesoderm formation with altered somitogenesis and gene expression (Pikulkaew *et al.* 2011, Nesan *et al.* 2012).

Moreover, transcriptomic analysis upon transient silencing of the receptor has been a powerful tool to potentially reveal direct and indirect interactors of the pathways involved in GC-Gr activity, as well as their relevance during embryogenesis. This approach allowed to identify Gr as a negative regulator of bone morphogenetic protein (BMP) signaling pathways, as demonstrated by downregulation of *bmp2a*, *bmp2b* and *bmp4* (Nesan *et al.* 2012, Nesan & Vijayan 2013). Additionally, according to the evidence that GCs can exert multiple functions, several other organs, including nervous system, heart and skeletal muscle, are also impacted during development (Nesan & Vijayan 2013).

These results not only confirm the previously described zebrafish morphants phenotypes, but also reflect the effects observed in mice (Cole *et al.* 1995), suggesting extensively overlapping Gr functions in the two model species. Thus, maternal liposoluble hormones already present in the oocyte and *gr* transcripts would represent a direct signaling link between the life experience of the mother and the capability of the progeny to cope with environmental and social challenges.

As maternal GC/Gr signaling is found to be essential for normal development both in teleosts and mammals, the exposure to an excess of cortisol causes specific effects on offspring development, thus demonstrating, beyond any doubt, that these hormones are both correlated with maternal hormonal condition during oogenesis and biologically active throughout the first stages of embryogenesis (reviewed by Moisiadis & Matthews 2014, Faght & Vijayan 2018b). Therefore, GC concentration needs to be finely regulated, since excess has negative effects on fetal development (Busada & Cidlowski 2017).

The approach of Wilson *et al.* (2016) to study the role of GC/Gr during development combined the *gr* knockdown with enrichment of egg cortisol and hypoxia, in order to analyze their combined effects on development, behavior and growth (Wilson *et al.* 2016). These experiments confirmed the central role of Gr in development as *gr* morphants showed impairments in hatch-rate, head-trunk angle and body length. Moreover, *gr* morphants appeared to be less responsive to tactile stimuli as well as less motile when compared to control larvae. *gr* morphants were raised to adulthood and showed significantly lower levels of cortisol when compared to controls, suggesting that *gr* knockdown during development affects the physiology of adult fish (Wilson *et al.* 2016). In addition, these manipulations showed how the variations of GC activity during early development while affecting embryonic processes, are also risk factors for diseases in adults. For example, knocked-down embryos had severe defects in heart morphology and functionality and these modifications in cardiac structures were not recovered in adult life, suggesting a connection between GCs and heart dysfunctions (Wilson *et al.* 2015, 2016).

The *gr* knockdown approach was adopted to study how hormones orchestrate acid-base balance and ion transport in fish, an issue recently reviewed by Guh & Hwang (2017). Among the hormones involved in these physiological processes, cortisol has been demonstrated to regulate the differentiation process of epidermal ionocytes, cells that are localized in the gills and involved in ion homeostasis. As a consequence of Gr transient silencing, the number of these cells was reduced (Cruz *et al.* 2013), and the expression of several transporters, such as epithelial calcium channel (ECC) which normally controls Ca²⁺ uptake (Lin *et al.* 2011) and sodium-chloride cotransporter (NCC) required for the absorption of Na⁺ (Kumai *et al.* 2012, Kwong & Perry 2013, Lin *et al.* 2016), were downregulated in *gr* morphants when compared to control larvae. Interestingly, knockdown experiments with splicing MOs for both Gr and Mr as well as

pharmacological selective inhibition or activation of these receptors using RU-486 and aldosterone, respectively (Kumai *et al.* 2012), revealed that, in teleost fish, all these osmoregulatory functions, are carried out by the Gr and not by the Mr, as far as development is concerned (Lin *et al.* 2015). These results were later confirmed with the analysis of osmoregulation in a medaka mineralocorticoid receptor (*mr*) mutant (Sakamoto *et al.* 2016).

Chatzopoulou *et al.* used the MOs for the characterization of Gr transcriptional activity of the two different splicing variants, Gr α and Gr β . In particular, specific morpholinos hindering *gr α* and *gr β* mRNA formation were used, and the resultant morphant transcriptomes were compared. The work demonstrates that Gr α regulates two partially overlapping gene clusters. Particularly, while a subset of Gr α -regulated genes is expressed under basal conditions, the treatment with dexamethasone (DEX) elicits transcription of a second subset of genes, with very weak effect on the genes of the first cluster (Chatzopoulou *et al.* 2015). Moreover, a luciferase reporter assay suggests a dominant-negative effect of Gr β on Gr α transcriptional activity

(Chatzopoulou *et al.* 2015) but this is not confirmed *in vivo*, and thus the function of Gr β isoform remains unclear (Chatzopoulou *et al.* 2017).

***In vivo* visualization of GC/Gr transcriptional activity in zebrafish**

A modern approach for the visualization of signaling pathways activities in metazoans takes advantage from the generation of transgenic lines expressing reporter genes under the control of the DNA responsive elements bound by the specific transcription factor under investigation (Fig. 3). These experimental models have been developed also for the *in vivo* visualization of GC/Gr transcriptional activity starting from 2012 with the generation of the GRIZLY (glucocorticoid responsive *in vivo* zebrafish luciferase assay) by Weger *et al.* (2012). This assay is based on the transgenic zebrafish reporter line *Tg(GRE:Luciferase)^{sb6}* or GRE-Luc, in which the expression of luciferase is driven by four concatemerized GREs. The specificity of this transgenic line was validated with both GC agonists

Figure 3

Main citations on GC-responsive transgenic lines.

and antagonists, and analyzed for the dose-dependence of luciferase activity. Notably, GRIZLY reports the increase of cortisol concentration in larvae upon exposure to osmotic stress thus demonstrating that this transgenic line allows to monitor stress-dependent cortisol production. This response was found to be detectable in 4-dpf (days post fertilization) transgenic larvae and significant at 5-dpf, thus confirming previous reports of [Alsop & Vijayan \(2008\)](#) regarding the ontogenesis of zebrafish stress response. The easy quantification of the luciferase activity makes this line a useful model for *in vivo* high-throughput screening of new GC agonist or antagonist drugs ([Weger *et al.* 2013](#)), or analysis of environmental toxins as demonstrated by the responsiveness of GRE:Luc line to tributyltin (TBT), a pollutant that is metabolically converted to the Gr antagonist dibutyltin (DBT) in the liver ([Ohhira *et al.* 2003](#)). As a matter of fact, while both TBT and DBT treatments inhibit luciferase activity in the GRE:Luc zebrafish line, only DBT is able to do it in AB.9 GRE:Luc cells, due to the lack of the specific metabolic process catalyzing its synthesis in this cellular model ([Weger *et al.* 2012](#)).

In 2014 [Krug *et al.*](#) generated the *Tg(6xGRE:EGFP,myl7:TagBFP)^{mm48}* transgenic line, called SR4G, a reporter model characterized by six composite GRE sequences upstream of the coding sequence of EGFP (enhanced green fluorescent protein). This reporter line was validated not only assessing the response to osmotic stress or treatment with GC agonists or antagonists, but also with fluorescence analysis of transgenic mosaic larvae TALENs engineered to target exon 2 of the *nr3c1* gene. As expected, the *gr* KO prevented the *EGFP* mRNA increase stimulated by a GC treatment. Furthermore, fluorescence levels increased after treatment with nicotine, used as an alternative activator of the HPI axis. Finally, *EGFP* mRNA levels mirror the oscillations of basal cortisol concentrations with the circadian rhythm ([Krug *et al.* 2014](#)).

Almost simultaneously, [Benato *et al.*](#) generated the *Tg(9GCRESV.U123:EGFP)^{ia20}* transgenic zebrafish line, from herein named *ia20*. The reporter plasmid inserted in the genome of this line contains nine GRE consensus sequences followed by the EGFP coding sequence. This reporter is different from the GRE:Luc both in the number of GRE repeats (which potentially can affect sensitivity) and in the spatial resolution of the signal when embryos/larvae, but also adult tissues, are analyzed in confocal microscopy. The high sensitivity of this transgenic line, confirmed by expression analysis of *EGFP* and *fkbp5* as a GC target gene, allows to visualize the response not only

to exogenous but also to endogenous GCs and to identify tissues/structures that are responsive to these hormones with very high spatial resolution. Similarly to the SR4G reporter line, the EGFP activity of *ia20* fish decreases with Gr knockdown and RU-486 treatment and shows variations in tissues specificity and intensity with respect to the light cycle ([Benato *et al.* 2014](#)).

After publication, all these reporter lines have found more applications in research: the GRIZLY assay was used to document the absence of 11 β -hydroxysteroid dehydrogenase type 1 activity in zebrafish ([Tsachaki *et al.* 2017](#)) and for *in vivo* detection of drugs able to interfere with the GC system.

The SR4G reporter demonstrated that handling zebrafish larvae during the execution of the intestinal transit assay induces an increase in cortisol secretion ([Brady *et al.* 2017](#)) and *in vivo* confirmed the interaction between methylmercury, an environmental contaminant, and the Gr, previously suggested by molecular dynamics simulations. Moreover, this interaction was found to have a role in the developmental neurotoxic effects determined by this molecule ([Spulber *et al.* 2018](#)). In addition, this line was adopted to monitor GC activity and confirm the loss of HPI functionality in the *mc2r* (adrenocorticotrophic hormone receptor) homozygous mutant fish ([Lee *et al.* 2019](#)).

Other applications of this reporter line were the visualization of (1) the hazard in the co-administration of aminoglycoside antibiotics with GCs as the latter can both increase the sensitivity of the lateral line to aminoglycoside damage ([Hayward *et al.* 2019](#)), (2) how GC/Gr complex blocks the functional recovery after spinal cord injury ([Nelson *et al.* 2019](#)).

The transgenic line *ia20* was similarly used to monitor GC activity in the first CRISPR/Cas9 zebrafish mutant line developed for Gr ([Facchinello *et al.* 2017](#), [Morbiato *et al.* 2019](#)), to study the cross-talk between GC/Gr and the hypoxic transcriptional responses ([Vettori *et al.* 2017](#)), to demonstrate how an increase of cortisol induced by stress impairs the regenerative potential of zebrafish heart ([Sallin & Jaźwińska 2016](#)) and to visualize the renin effects on pronephros development, as this line shows a robust expression of EGFP in fish kidney ([Schaeffer *et al.* 2019](#)).

Mutants of glucocorticoid receptors in zebrafish

The MO-induced knockdown of *gr* translation was a valuable tool to overcome the unavailability of targeted

mutagenesis techniques in *D. rerio* to study the Gr response in a living model organism. On the other hand, a knockdown strategy can have problems due to (1) off-target effects when the MO recognizes and works on genes other than those under consideration; (2) the lack of high precision and reproducibility in the amount of injected oligomers as well as (3) the problem of suitable experimental controls. Moreover, although this technique can help to elucidate the effects of a developmental transient knockdown on adult physiology (Wilson *et al.* 2015), it does not allow to analyze the biological effects due to the absence of *gr* in post-larval life.

Before the development of targeted KO techniques, the only available approaches to obtain zebrafish mutants was to perform laborious and time-consuming forward genetics assays. In 2005, a screen for zebrafish mutants of the vertebrate sensory system based on behavioral analyses, in this case on the visual background adaptation (VBA) assay, enabled the identification of a *gr* mutant line called s357 (Muto *et al.* 2005). The VBA assay is based on the neuroendocrine response determining the shrinkage of zebrafish melanocytes when larvae are exposed to bright background (Kurrasch *et al.* 2009). Nowadays, this test is used in strains defective in the synthesis of GCs for the preliminary selection of homozygous recessive larvae, in which this response is lost (Griffiths *et al.* 2012, Muto *et al.* 2013, Griffin *et al.* 2016, Eachus *et al.* 2017, Facchinello *et al.* 2017, Weger *et al.* 2018, Lee *et al.* 2019, Gans *et al.* 2020, Li *et al.* 2020, Marchi *et al.* 2020).

The *gr^{s357/s357}* mutant is characterized by a point mutation in the DBD that leads to a substitution of an arginine with a cysteine at the position 443. Hence, this mutant *gr* gene codes for a protein that is able to bind GCs and to translocate to the nucleus but cannot efficiently bind to GREs (Griffiths *et al.* 2012).

Given their overall morphological and physiological similarities compared to WT fish, *gr^{s357/s357}* mutants present higher chronic levels of cortisol both at larval and adult stages (Griffiths *et al.* 2012, Ziv *et al.* 2013). Furthermore, transcript levels of corticotropin-releasing hormone (*crh*) and proopiomelanocortin A (*pomca*), measured with qRT-PCR and *in situ* hybridization, are significantly higher in mutants when compared to WT siblings. All these data confirm the loss of HPI negative feedback loop, activated in WT fish by Gr- and GRE-mediated transcriptional regulation. Therefore, the *gr^{s357/s357}* zebrafish line was employed as a model to study HPI hyperactivation and development of depression, a condition that is also associated with GCs in humans; it is due to functional (GC resistance) or expressional changes in the Gr

(Baumeister *et al.* 2016). With respect to both WT larvae and adults, mutants respond with a reduced exploratory behavior and impaired habituation to stressful conditions, like new tanks, and startle response (Ziv *et al.* 2013). *gr^{s357/s357}* larvae show also a lower spontaneous activity (Griffiths *et al.* 2012). Interestingly, the anxiolytic drug diazepam and the antidepressant molecule fluoxetine, a selective serotonin reuptake inhibitor, are able to reduce the depression-like mutant behavior, possibly through modulation of HPI axis hyperactivity (Ziv *et al.* 2013). On the other hand, DEX treatment cannot induce a repression of the stress markers in mutant fish, a fact consistent with the abrogation of the negative feedback loop in *gr^{s357/s357}*.

These results point out the evolutionary conservation of the neuroendocrine circuits of stress among Vertebrates while suggesting the huge potential of zebrafish *gr* mutant lines for the screening of new pharmacological molecules for anti-depression and affective disorders therapies.

In addition, Muto *et al.* (2013) found that light adaptation is disturbed in the retina of *gr^{s357/s357}* larvae as they display behavioral delays when suddenly re-exposed to light after a dark period, as well as some changes in gene expression in the dopaminergic signaling axis, a key neuromodulator of the retinal network (Muto *et al.* 2013). Since its identification, the *gr^{s357/s357}* mutant line has been used by several research groups to analyze the regulation of sleep/wake states by different neuropeptides like neuromedin U (Chiu *et al.* 2016) or prokinectin 2 (Chen *et al.* 2017) or the neuroendocrine mechanisms involved in the perception of food (Filosa *et al.* 2016). Other applications of this line are listed in Fig. 4, which covers a considerable number of different physiological processes and underlies the value of the zebrafish model (Kwan *et al.* 2016, Brun *et al.* 2019, Mosser *et al.* 2019, Palstra *et al.* 2019, Sireeni *et al.* 2020).

The design of CRISPR/Cas9 targeted mutagenesis protocols and their application, with the subsequent possibility to make reverse genetics studies in zebrafish, was a breakthrough in the development of mutants (Hwang *et al.* 2013). This technique allowed Facchinello *et al.* (2017) to produce the *gr^{ia30}* mutation, the first zebrafish *gr* null allele. Since then, many zebrafish mutant lines have been generated targeting different positions of the protein as summarized in Fig. 5.

The *gr^{ia30}* contains a 5-nucleotide insertion in the second exon of the gene, which results in a frameshift mutation leading to the generation of a premature stop codon. The *gr^{ia30/ia30}* mutants are viable and fertile, although their offspring showed a reduced survival rate when compared to both *gr^{s357/s357}* and WT.

Genome editing
<p>one-cell embryo</p> <p>crRNA tracrRNA</p> <p>Cas9</p> <p>Mutant founder</p> <p>Mutant stable lines</p>		
Results	Source	Allele
<p>HPI axis, stress response and behaviour:</p> <p>Gr KO results in chronically high cortisol concentration Gr is essential for the correct response to stress</p> <p>The absence of a functional Gr leads to a depression-like behaviour that can be rescued by fluoxetine. The anxiogenic and depression-like behaviour due to loss of Gr signalling develops after the larval stages.</p> <p>Gr and Mr are both required to control HPI axis activity and work through different mechanisms. Gr, but not Mr, is involved in correct responses to different stressors like light stimuli and salinity</p> <p>Mutants for Cyp21a2, Cyp11a2, Fdx1b show a GCs deficient phenotype and can be used as a model for glucocorticoid-deficiency</p>	<p>Griffiths <i>et al.</i> (2012) Facchinello <i>et al.</i> (2017) Faught & Vijayan (2018a)</p> <p>Ziv <i>et al.</i> (2013) Sireeni <i>et al.</i> (2020)</p> <p>Faught & Vijayan (2018a) Lee <i>et al.</i> 2018</p> <p>Griffin <i>et al.</i> (2016) Eachus <i>et al.</i> (2017) Oakes <i>et al.</i> (2019) Li <i>et al.</i> (2020)</p>	<p><i>gr</i>^{s357} <i>gr</i>^{ia30} <i>gr</i>^{ca401} <i>mr</i>^{ca402} <i>gr</i>^{mn61} <i>gr</i>^{mn62} <i>gr</i>^{mn63} <i>gr</i>^{mn64} <i>gr</i>^{mn65} <i>mr</i>^{mn66} <i>mr</i>^{mn67}</p> <p><i>cyp21a2</i>^{uob2122} <i>cyp21a2</i>^{uob2123} <i>fdx1b</i>^{uob205} <i>cyp11a2</i>^{-/-}</p>
<p>Hypoxia response:</p> <p>GCs determine pseudohypoxia through DNA-binding independent mechanisms Hypoxia regulates steroidogenesis, but Gr transcriptional activity is dampened when Hif1 activity is induced Gr and Mr are both directly involved in hypoxic response</p>	<p>Vettori <i>et al.</i> (2017) Marchi <i>et al.</i> (2019)</p>	<p><i>gr</i>^{ia30} <i>gr</i>^{sh543}</p>
<p>Neurobiology and circadian rhythm:</p> <p>Gr is essential for regulation of retinal light adaptation Regulation of sleep/wake states by Neuromedin U and Prokineticin 2 do not require Gr. GCs regulate the amplitude of the circadian rhythm and is involved in the feeding state of zebrafish larvae GC/Gr is essential for feeding synchronization of circadian clock</p>	<p>Muto <i>et al.</i> (2013) Chiu <i>et al.</i> (2016) Chen <i>et al.</i> (2017) Mosser <i>et al.</i> (2019) Filosa <i>et al.</i> (2017) Morbiato <i>et al.</i> (2019)</p>	<p><i>gr</i>^{s357} <i>gr</i>^{ia30}</p>
<p>Muscle and bone development:</p> <p>GCs affect the white skeletal muscle transcriptome but not the exercise-enhanced growth Gr is fundamental for bone and cartilage correct formation.</p>	<p>Palstra <i>et al.</i> (2019) Jiang <i>et al.</i> (2020)</p>	<p><i>gr</i>^{s357} <i>gr</i>^{-/-}</p>
<p>Growth and metabolism:</p> <p>Gr controls larval growth and muscle glucose uptake Both Gr and Mr are involved in GCs-dependent lipid regulation during post-natal growth. Larval transcriptome shows that Mr regulates lipid synthesis and Gr activation promotes lipid catabolism Excess of cortisol suppresses growth Loss of Fdx1b blocks GCs' synthesis, that leads to dysregulation of many metabolic pathways, including reprogramming of glutamine metabolism</p>	<p>Faught & Vijayan (2019a) Faught & Vijayan (2019b) Faught & Vijayan (2020)</p> <p>Weger <i>et al.</i> (2018)</p>	<p><i>gr</i>^{ca401} <i>mr</i>^{ca402}</p> <p><i>fdx1b</i>^{uob205}</p>

Figure 4

Main citations and results on zebrafish mutant lines.

(Continued)

Figure 4 Continued.

Inflammation: GCs reduce neutrophil migration towards wound site and the inflammatory response Gr is essential for correct response to inflammation Some functions of Gr in inflammatory response appear to be DNA-binding independent <i>klf9</i> is a Gr target gene and encodes for a transcription factor that is involved in the expression of inflammatory genes Gr signalling is involved on embryonic hematopoietic stem and progenitor cell regulation	Chatzopoulou <i>et al.</i> (2016) Facchinello <i>et al.</i> (2017) Gans <i>et al.</i> (2020) Kwan <i>et al.</i> (2016)	<i>gr</i> ^{s357} <i>gr</i> ^{ia30} <i>gr</i> ³⁶⁹ <i>gr</i> ^{s357}
Toxicity: Developmental neurotoxicity of methylmercury is due to interference with Gr GCs sensitizes the hair cell of the lateral line to aminoglycoside toxicity Gr activation is involved in polystyrene nanoplastics adverse effects	Spulber <i>et al.</i> (2018) Hayward <i>et al.</i> (2019) Brun <i>et al.</i> (2019)	<i>gr</i> ^{s357}

To analyze GC signaling *in vivo*, the *gr^{ia30/ia30}* KO line was compared with the *gr^{s357/s357}* in *Tg(9GCRE-HSV.UI23:EGFP)* *ia20* transgenic background. Larvae of the two mutant lines show both a significant reduction in fluorescence and insensitivity to exogenous GC-based treatment, when compared to WT siblings, thus confirming the lack of the DNA-binding-dependent transcriptional activity. These data were also confirmed by qRT-PCR analysis of *fkbp5*, a GC target gene, whose expression is significantly reduced in both mutants and upregulated by DEX only in WT sibling larvae. As expected, also the *gr^{ia30/ia30}* null mutant line is characterized by high levels of whole-body cortisol, associated with overstimulated basal levels of *crh* and *pomca* transcripts and HPI axis unresponsiveness to a mechanical stressor (Facchinello *et al.* 2017).

Remarkably, analyses after inflammatory stimuli suggest that *gr^{s357/s357}* mutants differ from *gr^{ia30/ia30}*, possibly due to DNA-binding-independent activities of Gr, such as tethering with other transcription factors,

preserved only in *gr^{s357/s357}*. In particular, inflammation-related gene expression is stimulated in WT after dextran sulfate sodium (DSS) treatment, while the following DEX treatment can restore it to basal levels. In *gr^{ia30/ia30}* mutants, the expression of markers of inflammation does not change after DSS and DEX treatment, suggesting that the absence of Gr determines the inability to properly respond to inflammation. Notably, inflammation-related genes appeared to be upregulated by DSS in *gr^{s357/s357}* larvae when compared to untreated ones, even if their upregulation is lower than in treated WT larvae. Furthermore, DEX is able to restore basal conditions in *gr^{s357/s357}* revealing that this mutant line is able to respond to exogenous GCs, while *gr^{ia30/ia30}* is not (Facchinello *et al.* 2017). Although the inflammatory issue in *gr^{ia30/ia30}* needs to be analyzed more in details, the results obtained are consistent with the findings that the GC trans-repression of inflammatory responses, carried out by GR protein-protein interactions, is the preeminent mechanism used

Figure 5

Schematic representation of zebrafish glucocorticoid receptor organization. The figure highlights the position of the mutations in the *gr* mutant lines described in this paper and the experimental approach used to generate them.

to downregulate the activity of many transcription factors involved in the immune response such as AP-1 or NF- κ B (reviewed in [Ratman *et al.* 2013](#)). The maintenance of Gr tethering activities in *gr^{s357/s357}* is furthermore confirmed by the analysis of the crosstalk between GCs and hypoxic transcriptional responses: hypoxia-inducible factor (Hif) activation by GC-based treatment is still expressed in the mutant line with a missense mutation in its DNA binding domain (*gr^{s357/s357}*) but it is absent in the KO one ([Vettori *et al.* 2017](#)), a result further confirmed later on by Marchi *et al.* with the generation of another *gr* mutant line (*gr^{sh543}*) ([Marchi *et al.* 2020](#)).

In 2019, Morbiato *et al.* used the *gr^{ia30/ia30}* line to study circadian clock entrainment. Like the majority of living organisms, zebrafish have adapted their physiology to anticipate the changes of environmental conditions rhythmically occurring during the day. The molecular mechanisms underlying this adaptation are generally defined as ‘circadian clock’. In vertebrates, rhythmicity is given by multiple oscillatory networks that are orchestrated by a central oscillator, tuned by environmental synchronizers (*zeitgebers*) such as light:darkness cycle, temperature cycles and food availability. Whereas behavioral activity and clock gene expression are entrained by the light:darkness (LD) cycle, feeding entrainment is severely dampened in *gr^{ia30/ia30}* mutants, both in adults and in larvae, thus confirming the role of GC-mediated Gr signaling in this environmental synchronizer in a non-mammalian species. Moreover, mutants also show a significant reduction of period 2 (*per2*) expression, a crucial component of the circadian clock in fish. Additionally, expression of phosphoenolpyruvate carboxykinase 2 (*pck2*) and sterol regulatory element-binding protein 1 (*sreb1*) genes, which are involved in glucose and lipid metabolism, is altered in *gr^{ia30/ia30}* mutants when compared to WT. While the rhythmicity of *pck2* is completely abolished, *sreb1* rhythm undergoes a phase shift, anticipating the acrophase of its expression. The study confirms that, like in mammals ([Challet 2019](#)), in non-mammalian vertebrates GC/Gr may function as an endocrine signal able to regulate feeding synchronization with circadian clocks ([Morbiato *et al.* 2019](#)).

To distinguish the different physiological roles of Gr and Mr in the zebrafish model, [Faught & Vijayan \(2018a\)](#) produced and compared *gr* (*gr^{ca401/ca401}*) and *mr* (*mr^{ca402/ca402}*) mutant lines. Although the transcript levels of *gr* and *mr* in the corresponding mutant lines are not reduced with respect to WT, as normally happens for the activation of the nonsense-mediated mRNA decay (NMD) machinery ([Chang *et al.* 2007](#)), the loss of proteins

is confirmed by Western blotting. Furthermore, *gr^{ca401/ca401}* mutants show the physiological features due to the Gr silencing, such as high level of cortisol and deregulation of the HPI axis. On the contrary, in the *mr* mutant line cortisol basal level is normal. Comparative analysis of these mutant lines demonstrates that both Gr and Mr are involved in the regulation of stress axis activation but with different functions: while *gr^{ca401/ca401}* mutants are unable to produce a stress response, in *mr^{ca402/ca402}* the reaction to acute stress is delayed. Notably, because the loss of Mr results in behavioral changes which are not rescued by the administration of exogenous GCs, both Gr and Mr are required and have complementary activities in stress-related behavior ([Faught & Vijayan 2018a](#)). This response, mediated by MR and GR, also occurs in mammals, besides the osmoregulatory process ([Joëls & de Kloet 2017](#)).

Since GC/Gr signaling exerts a significant role for energy and metabolic homeostasis in muscle, [Faught & Vijayan \(2019a\)](#) analyzed the outcomes of Gr KO in the *gr^{ca401/ca401}* mutant line on muscle physiology. In agreement with muscle-specific GR mutant mouse models (reviewed by [Whirledge & DeFranco 2018](#)), also these hypercortisolemic zebrafish *gr* mutants have higher body mass due to an increase of lipid and protein, but not of carbohydrate, content. Moreover, while the glucose level increases in stressed WT fish, this response is hampered in mutants that show also a higher permeability to glucose in muscle but not in liver. This observation matches with the higher activity in mutants of hexokinase, a crucial enzyme in glucose muscular uptake and utilization. Accordingly, loss of body weight during fasting was reduced in mutants. Thus, GC/Gr was identified as a growth suppressor in fish, potentially acting on protein catabolism ([Faught & Vijayan 2019a](#)). These results were later confirmed with the analysis of corticosteroid receptors (CRs) specific functions during zebrafish larval growth ([Faught & Vijayan 2020](#)). According to these authors, both Gr and Mr are involved in postnatal growth regulation. Growth suppression due to GC excess results from Gr mediated upregulation of muscle proteolytic markers like muscle RING-finger protein 1 (*murf1*) and regulated in development and DNA damage responses 1 (*redd1*), although the activation of Mr is also required for this catabolic effect. While stimulation of protein synthesis, important for postnatal growth, is promoted by Mr, Gr seems to play its catabolic functions when GCs increase ([Faught & Vijayan 2020](#)).

A similar comparative experimental setting was conceived by [Faught & Vijayan \(2019b\)](#) to study the roles of Gr and Mr, as well as their specific interaction,

on lipid metabolism, under basal and stressful conditions (cortisol treatment). In their work, the authors showed that Mr, the only receptor present in Gr mutants, plays a fundamental role in the biosynthesis of triglycerides and in lipid accumulation by downregulating the transcription of the enzyme lipoprotein lipase, thus explaining the increase of adipose tissue previously reported in Gr mutant lines (Facchinello *et al.* 2017, Faught & Vijayan 2019a). Moreover, similarly to what happens in muscle, transcriptome analysis of mutant larvae demonstrates how Mr and Gr control the transcription of different and specific genes, with Mr more involved in lipid synthesis while Gr in lipid catabolism (Faught & Vijayan 2019a).

Lee *et al.* analyzed the correlation between rapid locomotor responses following different acute stressors and the activity of the HPI axis (Lee *et al.* 2019). These behavioral responses are different depending on the stressor and are abolished in zebrafish mutant lines that lack ACTH receptor (*mc2r*), which is crucial for the synthesis of GCs. The authors conclude that an intact HPI axis is required for a rapid locomotor response. Moreover, analysis of new *gr* and *mr* mutant lines, together with the use of specific antagonists suggest that Gr and not Mr is the receptor involved in these responses (Lee *et al.* 2019). This result contrasts with the Faught & Vijayan (2018a) hypothesis of a complementary role of Mr and Gr receptors in stress response. Indeed, the authors conclude that further analyses are required to better clarify the role of Mr in stress and rapid locomotor response (Lee *et al.* 2019).

Recently, Gans *et al.* (2020), analyzed the transcriptomes of WT and *gr* mutants (*gr*^{369/369}) upon chronic cortisol treatment. The outcome of a transcriptional activity in WT larvae similar to that of *gr*^{369/369} when chronically treated with cortisol, is explained by the authors with the development of Gr resistance. Moreover, they identified the transcription factor Kruppel-like factor 9 (Klf9), for which they generated a mutant line, as a crucial player in the regulation of the transcriptional response to GCs (Gans *et al.* 2020).

Finally, Jiang *et al.* (2020) generated a *gr* mutant line to study bone mineralization and the transcriptional control of genes involved in this process, as well as to clarify the role of GC/Gr in the establishment of osteoporosis and predisposition to bone fracture, one of the major side effects of chronic therapeutic treatment with GCs. Analysis of their mutant line shows that *gr* KO negatively affects the cartilage development, reduces the bone mineralization area, and modify the expression of bone-related genes, such as alkaline phosphatase (*alp*)

and acid phosphatase 5a (*acp5a*). Differently from other papers that report how GCs downregulated the expression of matrix metalloproteinases (*mmp9* and *mmp13*) after their upregulation during the inflammatory response (Tuckermann *et al.* 1999, Eberhardt *et al.* 2002, Chatzopoulou *et al.* 2016, Facchinello *et al.* 2017), these authors found an upregulation of *mmp* genes by treatment with prednisolone, a synthetic GC. Although these differences could be due to the GCs used, their concentration, length of treatment as well as developmental stage, an in-deep analysis of transcriptional control of these proteins by GCs could help to better elucidate the role of GCs in the transcriptional regulation of *mmp* genes.

To our knowledge, only three null mutant lines for corticosteroid receptors were developed so far in teleost models other than zebrafish, two in medaka and one in tilapia. The first medaka mutant line was generated for Mr. This receptor is prevalently expressed in brain and eyes, and analysis of the mutant line rules out the involvement of Mr in body fluid regulation, confirming results previously obtained by using morpholinos (Sakamoto *et al.* 2016). Since *mr* mutants show abnormal responses to visual stimuli, the authors suggest a major role for Mr on behavior (Sakamoto *et al.* 2016), in agreement with zebrafish *mr* mutant results (Faught & Vijayan 2018a).

The second medaka mutant line was employed to study the role of Gr in osteoblast and osteoclast function by KO of the paralogue gene *gr2*, since this was considered more responsive to GCs (Azetsu *et al.* 2019). Exploiting a transgenic background for osteoclasts and osteoblasts visualization and the *gr2* mutation, these authors show that the delay of fracture healing that follows chronic GC treatment is due to the suppression of osteoclast recruitment and accumulation of osteoblasts at the healing site (Azetsu *et al.* 2019).

Although the results obtained with *gr* and *mr* knock down and the *mr* medaka mutant line converge toward a clear role of Gr on acid-base balance and ion transport, to our knowledge, this evidence has not been confirmed with specific experiments or expression analyses performed on the different *gr* mutant lines available. However, despite the lack of this crucial regulation, *gr* mutants are viable. This suggests the activation of compensatory mechanisms based on complementary pathways or the upregulation of paralogue or related genes whose products can compensate for the loss of Gr. As a matter of fact, Gr and Mr proteins are closely related and derive from the gene duplication process of an ancestral corticoid receptor (CR) (Baker & Katsu 2017).

To compensate for the loss of *mr*, medaka *mr* KO results in an increase of *gr1* expression (one of the two paralogue *gr* genes present in this teleost species) in brain and eyes but not in the osmoregulatory organs (Sakamoto *et al.* 2017), confirming the lack of Mr activities in ion balance physiology. In zebrafish, compensation between CRs can be detected in the *gr^{ia30}* mutant line that shows a four-times increase of *mr* expression in 5-dpf larvae. On the other side, an even more intense increase of *gr* transcripts was measured in the *mr^{ia32}* mutant line (A Dinarello & L Dalla Valle, unpublished observations).

Another key point of Gr function is related to reproduction, since GCs have been proposed to play a crucial role in this process both in mammals (Whirledge & Cidlowski 2017) and in fish (Faught & Vijayan 2018b). Zebrafish *gr* homozygous mutants are genetically viable, but their fertility is significantly reduced, in particular with aging (Facchinello *et al.* 2017), a result confirmed also by Gans *et al.* (2020). As hypothesised by Faught & Vijayan (2018b), our analysis on *gr^{ia30/ia30}* females confirms that GCs and Gr control ovarian maturation and the ovulation process. In addition, we found that the complex is also deeply involved in macromolecular composition and quality of the oocyte cytoplasm (F Maradonna, G Gioacchini, V Notarstefano, CM Fontana, F Citton, L Dalla Valle, E Giorgini & O Carnevali, unpublished observations).

The study of GC function can also be addressed by blocking their synthesis through interference at different steps of steroidogenesis, the highly articulated and complex metabolic pathway supported by the enzymes involved in the biosynthesis of steroid hormones. Steroidogenesis takes place in the classical steroidogenic organs like gonads, adrenals and placenta, as well as in many peripheral tissues. Although some of these bioactive steroids can be produced *de novo* in small amounts, local steroids usually derive from the metabolism of circulating inactive precursors (Miller 2017).

The steroidogenic enzymes can be divided into two major classes: the first constituted by hydroxysteroid dehydrogenases (HSDs) and the second constituted by cytochrome P450, the latter in turn divided into type I, mitochondria integral proteins, and type II, targeted to the endoplasmic reticulum (Miller 2017). In particular, cortisol synthesis from cholesterol requires the activity of three P450 steroid hydrolases, as reported in Fig. 6, where the enzymes involved in GC synthesis, and already targeted in zebrafish with genetic KO, are underlined.

In addition, all P450 enzymes (also called CYP) require reducing equivalents to catalyze their reactions, hence, steroid synthesis depends on their availability. Electrons for mitochondrial P450 enzymes, (type I enzymes, Cyp11a1, Cyp11a2 and Cyp11c1 in zebrafish) are provided by NADPH through two electron transfer proteins – ferredoxin reductase and ferredoxin – whereas P450 type II enzymes depend on the electron donor enzyme cytochrome P450 oxidoreductase (POR) (Miller *et al.* 2017).

Zebrafish has two ferredoxin paralogs, Fdx1 and Fdx1b. Silencing of the paralog *fdx1b* gene, mainly expressed in steroidogenic tissues, was performed by Griffin *et al.* in 2016. *fdx1b^{UOB205/UOB205}* larvae showed very limited GC synthesis and the typical physiological features due to impairment of GC signaling, already described for *gr* mutant lines. Knockdown of the other paralog gene, *fdx1*, severely impairs early zebrafish development, thus preventing the analysis of its effects on the steroidogenic pathway (Griffin *et al.* 2016). Subsequent analysis by Weger *et al.* (2018) of the *fdx1b* cortisol-deficient zebrafish line focused both on metabolic changes and on the expression of genes involved in metabolic pathways. Results were then compared with those obtained both from a zebrafish model of secondary adrenal insufficiency, the *chokh/rx3* strong mutant fish line that presents corticotrope cells deficiency associated with reduced cortisol levels (Dickmeis *et al.* 2007), and from human patients affected by primary insufficiency. Among the effects found in *fdx1b* mutants, hence regulated by GCs, there are: a reduced transcription of liver and intestine glutaminases (*gls2a* and *gsl2b*), resulting in the increase of glutamine levels, reduction of antioxidant glutathione synthesis and consequent increase of oxidative stress markers, and post-transcriptional regulation of purine synthesis enzymes. Based on these results, the *fdx1b* mutant line has been proposed as an animal model for the study of human pathologies linked to GC deficiency, and also for the identification of biomarkers for the improvement of adrenal insufficiency diagnosis.

Adult *fdx1b* mutant males show defects linked to sexual determination and reproduction such as feminization of secondary sexual characteristics, disorganization of testis morphology, impairment of sperm, breeding behavior, and infertility. Androgen production decreases, further confirming that Fdx1b is required for the activity of the 11 β -hydroxylase, the enzyme encoded by the *cyp11c1* gene (Oakes *et al.* 2019). This enzyme catalyzes the conversion of 11-deoxycortisol into cortisol but also

Figure 6

Schematic representation of the steroidogenic pathway. The biosynthetic pathway is not complete but focused on the enzymes required for GC synthesis. Colored boxes highlight the mutated proteins.

converts testosterone and androstenedione to 11β -OH-testosterone and 11β -OH-androstenedione, two precursors of 11-ketotestosterone, the hormone that binds the androgen receptor (Tokarz *et al.* 2015) (Fig. 6).

The steroidogenic pathway for GC synthesis has been blocked also by KO of the *cyp21a2* gene that encodes the 21-hydroxylase enzyme, essential for the synthesis of GC hormones (Eachus *et al.* 2017). This block determines not only cortisol deficiency but also the accumulation of cortisol precursor like 17-hydroxyprogesterone and 21-deoxycortisol. Unfortunately, the outcomes of this effect have not been analyzed.

The *cyp11a2* zebrafish mutant line generated by Li *et al.* (2020) presents GC deficiency and all-male fish showing a moderately feminized phenotype. While *cyp11a1* is present as maternal transcripts and expressed until 24 hpf (hours post-fertilization), *cyp11a2* expression starts at 32 hpf (Parajes *et al.* 2013). This enzyme is essential for GC synthesis in larvae as well as for interrenal and gonadal steroidogenesis in adults (Li *et al.* 2020). Adult homozygous mutants are sterile and have disorganized testis and spermatogenesis as well as a significant reduction of male sex steroid production. However since its paralogue gene *cyp11a1* is present and active at least during early development an analysis of a possible genetic compensation could be an interesting issue to focus on.

The last P450 enzyme involved in cortisol synthesis is Cyp11c1, whose KO was performed both in zebrafish and in Nile tilapia (*Oreochromis niloticus*) (Zhang *et al.* 2020, Zheng *et al.* 2020). Since this enzyme is required for the synthesis of both cortisol and 11-ketotestosterone, these two mutants show delayed spermatogenesis, reduced sperm production and alteration of testis morphology. Notably, the spermatogenesis-related phenotype is

rescued by treatment with 11-ketotestosterone in both species. Spermatogenesis is naturally recovered in the Nile tilapia at 6 months post-fertilization, probably by the compensatory activity of testosterone whose serum concentration increases in these mutants (Zheng *et al.* 2020). Moreover, analysis of female reproduction confirms the role of cortisol on zebrafish oocyte maturation and ovulation (Zhang *et al.* 2020), already shown in the *gr^{ia30/ia30}* mutant line.

Conclusions and future directions

The review by Schaaf *et al.*, published in 2009, while underlining the potentiality of zebrafish for GC and Gr research, also described the restricted number of tools available at that time (Schaaf *et al.* 2009).

Now this problem seems to be somehow solved with the application of MO knockdown on *gr* expression, the generation of specific transgenic zebrafish reporter lines and, more recently, the availability of a great number of new *gr* as well as other GC-related mutant lines. These advances have promoted, in a limited amount of time, an exponential growth of the scientific results obtained with this animal model, hence, increasing significantly our knowledge about GC biology.

Morpholino knockdown of zebrafish *gr* revealed that the GC/Gr complex regulates the development of many structures and processes like mesoderm formation and heart development (Nesan *et al.* 2012, Wilson *et al.* 2015), BMP signaling pathway (Nesan & Vijayan 2012, 2013), behavior and growth (Wilson *et al.* 2016). Furthermore, maternal *gr* transcripts have been proven to have a pivotal role in the correct larval development while zygotic *gr*

mRNA seems to exert a limited contribution (Pikulkaew *et al.* 2011). This result highlights the importance of a correct concentration of maternal cortisol during development, not only in zebrafish but also in humans (Moisiadis & Matthews 2014), and how zebrafish can be a useful model for the study of negative effects caused by either positive or negative variations of this hormone.

Ion balance was also investigated in zebrafish *gr* morphants confirming the fundamental role of Gr rather than Mr in both ionocyte differentiation as well as transcription of ion channels and cotransporters (Lin *et al.* 2011, Cruz *et al.* 2013, Kumai *et al.* 2012).

Living biosensors for Gr transcriptional activity with different and specific features have been generated and used to analyze *in vivo* the alteration of GC/Gr transcriptional activity in different conditions, or to screen for molecules able to interfere with GC signaling (Weger *et al.* 2012, Benato *et al.* 2014, Krug *et al.* 2014).

The first zebrafish *gr* mutant line, identified by screening of a mutant library, was the *gr^{s357/s357}* line. In this zebrafish line, widely used in particular to study zebrafish neurobiology and behavior linked to GC activities, a missense mutation abolishes any DNA dependent transcriptional activity of Gr (Ziv *et al.* 2013).

However, the most prominent advance in GC research came after application of genome editing approaches to obtain null *gr* mutant lines. In the last 3 years, many research groups generated *gr* mutant zebrafish lines and applied them to improve our knowledge on many of the physiological processes in which these steroids are involved, and to elucidate the different roles played by Gr and Mr (Fig. 4). It is worth noting that *gr* morphants show a slightly more severe phenotype compared with mutants. This result, widely discussed in Rossi *et al.* (2015) and in Eve *et al.* (2017), can be due to several causes such as off-target effects of MOs that can worsen morphant larvae phenotype or by the activation, in mutants but not in morphants, of genetic compensation mechanisms mediated by the transcriptional activation of paralog genes. Interestingly, a recent publication by El-Brolosy *et al.* (2019) revealed that in murine and zebrafish mutants the degradation of mutated transcripts by means of RNA decay triggers a transcriptional adaptation. Instead, mutants unable to degrade mutated mRNAs display a stronger phenotype.

The activation of genetic compensation, by allowing the achievement of adult life, gives the possibility to study the effects of gene silencing on adult organisms and, last but not least, it enables the analysis of alternative pathways useful in the search for new therapies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

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