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Review

Corticosteroid signaling in frog metamorphosis

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ABSTRACT

Stress in fetal and larval life can impact later health and fitness in humans and wildlife. Long-term effects of early life stress are mediated by altered stress physiology induced during the process of relaying environmental effects on development. Amphibian metamorphosis has been an important model system to study the role of hormones in development in an environmental context. Thyroid hormone (TH) is necessary and sufficient to initiate the dramatic morphological and physiological changes of metamorphosis, but TH alone is insufficient to complete metamorphosis. Other hormones, importantly corticosteroid hormones (CSs), influence the timing and nature of post-embryonic development. Stressors or treatments with CSs delay or accelerate metamorphic change, depending on the developmental stage of treatment. Also, TH and CSs have synergistic, antagonistic, and independent effects on gene regulation. Importantly, the identity of the endogenous corticosteroid hormone or receptor underlying any gene induction or remodeling event has not been determined. Levels of both CSs, corticosterone and aldosterone, peak at metamorphic climax, and the corticosteroid receptors, glucocorticoid and mineralocorticoid receptors, have wide expression distribution among tadpole tissues. Conclusive experiments to identify the endogenous players have been elusive due to difficulties in experimental control of corticosteroid production and signaling. Current data are consistent with the hypothesis that the two CSs and their receptors serve largely overlapping functions in regulating metamorphosis and synergy with TH. Knowledge of the endogenous players is critical to understanding the basic mechanisms and significance of corticosteroid action in regulating post-embryonic development in environmental contexts.

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1. Introduction

Corticosteroid hormones (CSs) are highly conserved hormonal mediators of the stress response across vertebrates (Ballard, 1986; Nesan and Vijayan, 2013). CSs are also critical vertebrate developmental hormones regulating (1) organ maturation in brain, lungs, pancreas, and other organs and tissues, (2) developmental transitions of metamorphosis in fish and amphibians, hatching in chicks, and birth in mammals, and (3) long-term effects of stress during development, such as survival and fecundity in frogs, growth and coping styles in birds, and cardiovascular and metabolic health in humans (Braun et al., 2013; Crino et al., 2014; Hu et al., 2008; Nesan and Vijayan, 2013; Schoech et al., 2012) These corticosteroid-dependent developmental processes and their interactions with the environment are complex, and the signaling mechanisms and downstream effectors underlying the effects of stressors on development are largely unknown.

Progress to identify the endogenous players of corticosteroid action in development has been made using knockout animals lacking CSs or their receptors in mouse and zebrafish. Glucocorticoid receptor knockout mice and offspring of mice deficient in adrenocorticotropin (pituitary hormone required for glucocorticoid synthesis) all die just after birth due to lung atelectasis from complete lack of glucocorticoid signaling (Cole et al., 2001; Saedler and Hochgeschwender, 2011). Lack of aldosterone synthase results in 70% survival after weaning (Lee et al., 2005), but MR knockout mice all die of salt wasting around weaning (Bleich et al., 1999). Even though the acute causes of death are known and the survivors serve as good models for studying adult deficiencies, the numerous pleiotropic actions of these hormones affecting various aspects of fetal development are understudied. Embryonic effects of glucocorticoid receptor have been studied in more detail in fish. Knockdown of glucocorticoid receptor translation by morpholinos showed internal and external morphological defects in zebrafish (Nesan et al., 2012; Pikulkaew et al., 2011), as well as impaired ionocyte development in medaka (Trayer et al., 2013). Genetic disruption of adrenocorticotropin identified roles for this hormone and its receptor in the development of interrenal tissue (To et al., 2007).

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Genetic manipulations of genes involved in corticosteroid signaling is advanced in mouse and fish compared to tadpoles. However, fetal endocrine manipulations used to elucidate developmental roles of hormones are often challenging because of confounding maternal influences in mice. Also, potential developmental roles of mineralocorticoids are not possible in fish, due to their lack this class of steroid. Frog metamorphosis has been a leading model in developmental endocrinology (Shi, 2009) due to the complete dependence of dramatic morphological changes on thyroid hormone and the occurrence of free-living tadpoles rather than uterus-enclosed embryos. In addition, tadpole growth and development are especially sensitive to environmental stressors, which include climate change, endocrine disruption, and habitat disturbance (Hayes et al., 2010). On the other hand, fundamental aspects of corticosteroid physiology in development have not been elucidated in frogs.

Most metamorphosis research has focused on the role of thyroid hormone (TH) because it is necessary and sufficient to initiate metamorphic events (Brown and Cai, 2007; Dodd and Dodd, 1976; Shi, 1999). The involvement of CSs in metamorphosis is more complex than TH, and understandably, the roles of CSs and their receptors on gene regulation and metamorphic transformation are less clear. The main CSs produced by the interrenals in frogs are the glucocorticoid corticosterone (CORT, the frog stress hormone) and aldosterone (ALDO, the frog mineralocorticoid hormone) as determined by *in vitro* biosynthesis studies in tadpoles and adults of *Lithobates catesbianus* and *Xenopus laevis* (Carstensen et al., 1961; Chan and Edwards, 1970; Jolivet-Jaudet and Leloup-Hatey, 1984; Ulick and Solomon, 1960) Previous excellent reviews have included sections on corticosteroids in metamorphosis (Denver, 2009; Denver, 2013; Dodd and Dodd, 1976; Kaltenbach, 1996; Wada, 2008; White and Nicoll, 1981). The current review outlines the central control of corticosteroid production and effects of CSs on metamorphosis and then critically examines the knowns and unknowns concerning the endogenous hormones and receptors involved in corticosteroid signaling regulating metamorphic transformation.

2. Hypothalamus–pituitary–interrenal and hypothalamus–pituitary–thyroid axes

Metamorphosis begins when TH first enters circulation, causing premetamorphic tadpoles to enter prometamorphosis (Etkin, 1964; Leloup and Buscaglia, 1977). When circulating levels of TH and CSs reach their peak, tadpoles experience metamorphic climax, a period of dramatic morphological remodeling and physiological changes. TH levels return to baseline at the end of metamorphosis upon complete tail resorption. Brain processing of external environmental signals from predators, water availability, food, temperature as well as internal signals such as energy balance determines the levels of TH and CSs produced by the hypothalamus–pituitary–interrenal and hypothalamus–pituitary–thyroid axes and thus determines the timing of and size at metamorphosis (Denver et al., 2009). Synthesis and release of TH by the thyroid glands and CSs by interrenal glands (homologous to mammalian adrenal glands) are induced by the pituitary hormones thyrotropin (thyroid stimulating hormone, TSH) and corticotropin (adrenocorticotropic hormone, ACTH), respectively (Denver et al., 2009). The release of these pituitary hormones is under the influence of the hypothalamus. In adult frogs, thyrotropin releasing hormone (TRH) stimulates TSH release (Darras and Kuhn, 1982), and corticotropin releasing hormone (CRH) stimulates the release of TSH and ACTH (Kuhn et al., 1998; Tonon et al., 1986). In tadpoles and axolotls, CRH regulates the release of both TSH and ACTH, but TRH control over TSH release develops only after metamorphic climax (Denver, 1996; Jacobs et al., 1988; Kühn et al., 2005) TH exerts negative feedback on the hypothalamus–pituitary–thyroid axis throughout the larval period (Manzon and Denver, 2004), but negative feedback by CSs on CRH or ACTH has not been shown in tadpoles.

3. Effects of CSs on metamorphic progression

Early reports revealed that CSs (both CORT and ALDO) accelerate TH-induced metamorphic changes in tadpoles but have no metamorphic effect in the absence of TH in *Bufo bufo*, *Babelomurex japonicus*, and *X. laevis* (Bock, 1938; Frieden and Naile, 1955; Gasche, 1945; Kobayashi, 1958). Sub-epidermal implantation of cortisol or desoxycorticosterone acetate (a mineralocorticoid) pellets caused local tail resorption only in the vicinity of the pellet if sub-threshold doses of TH were also included in the pellet in *Lithobates pipiens* (Kaltenbach, 1958). Later reports showed that TH induction of tail shrinkage, limb outgrowth, head shape change, gut tube shortening, skin keratin expression, and hepatic enzyme carbamoyl-phosphate synthase activity were increased upon co-treatment with CORT in *X. laevis* (Galton, 1990; Gray and Janssens, 1990; Shimizu-Nishikawa and Miller, 1992; Wright et al., 1994). *In vitro* studies on cultured tail tips showed CORT and ALDO accelerated TH-induced tail shrinkage in *B. japonicus* and *X. laevis* (Bonett et al., 2010; Gray and Janssens, 1990; Kikuyama et al., 1983). In contrast, during premetamorphosis (when endogenous TH is low or absent), exogenous treatment with CORT, cortisol, dexamethasone (a glucocorticoid receptor-specific agonist), ALDO, and desoxycorticosterone acetate inhibited growth and development in *Anaxyrus boreas*, *B. japonicus*, and *X. laevis* (Hayes, 1995; Kobayashi, 1958; Leloup-hatey et al., 1990; Lorenz et al., 2009; Rapola, 1962; Wright et al., 1994) In prometamorphosis (circulating TH present), treatment with CORT or desoxycorticosterone acetate alone increased metamorphic rate in *A. boreas* and *B. japonicus* (Hayes, 1995; Kobayashi, 1958). In *X. laevis*, CORT treatment during prometamorphosis still blocked TH-induced tail resorption and forelimb emergence, though gill resorption still occurred, and ALDO had no effect (Leloup-Hatey et al., 1990). In the CORT-treated tadpoles, TH levels in plasma declined consistent with inhibition of metamorphosis. Rearing conditions that induce stress and increase CORT content during prometamorphosis resulted in an increased rate of metamorphosis in spadefoot toads (Denver, 1998; Kulkarni et al., 2011; Newman, 1989). In summary, exogenous treatment of the two classes of CSs (glucocorticoid and mineralocorticoid) have generally comparable effects on growth and development in tadpoles.

Despite clear effects on growth and development, exogenous hormone treatments per se do not reveal the endogenous actors of corticosteroid physiology. Experiments blocking corticosteroid signaling are required to elucidate such roles. Hypophysectomized (pituitary removed) tadpoles of *Alytes obstetricans* did not undergo metamorphosis (for lack of pituitary signal to make TH) but did initiate metamorphosis upon TH treatment. However, the tadpoles were unable to complete metamorphosis, unless ACTH was also given (Remy and Bounhiol, 1971). Tadpoles or tail tips treated with amphenone B (glucocorticoid synthesis inhibitor) showed reduced rate of induced metamorphosis (Kikuyama et al., 1982). Treating *A. boreas* tadpoles with metyrapone (another glucocorticoid synthesis inhibitor) caused 33% reduction in CORT, which resulted in reduced rate of hindlimb development but did not affect the rate of tail resorption (Hayes and Wu, 1995). Further work by Glennemeier and Denver showed that treatment with metyrapone reduced whole body CORT by 50% in *Lithobates pipiens* tadpoles but did not affect the rate of metamorphosis (Glennemeier and Denver, 2002b). ALDO was not measured even though the inhibitors also block aldosterone synthase activity. These experiments

demonstrate involvement of CORT and/or ALDO, but which one or both was not precisely identified.

In contrast to morphological studies, transcriptional regulation by CORT has received little attention. Detailed study of gene regulation by CORT during metamorphosis has been done for a single gene, *KLF9* (Krüpel-like factor 9) (Bonett et al., 2009) To date, one transcriptome-wide study examined the scope of CORT and TH and their interaction on gene regulation, but multiple research groups are currently conducting related RNA-seq studies on various organs. The transcriptome analysis was conducted on premetamorphic *X. tropicalis* tadpole tails harvested 18 h after treatment *in vivo* with 100 nM CORT, 10 nM T3, or CORT + T3 (Kulkarni and Buchholz, 2012). A total of 1968 genes were regulated by CORT. Hormone-regulated genes were clustered into patterns based on up- and down-regulation by one or both TH, CORT, and CORT/TH in all combinations. The pattern identified with the most genes was CORT and TH in synergy (22.5%). A surprisingly large number of genes (17%) manifested interactions between CORT and TH that were not synergy but various forms of antagonism. It is not clear how genes with antagonistic patterns of regulation relate to the apparently exclusively synergistic morphological effects of TH and CSs co-treatments. Another inhibitory action of CORT that occurs via central control is the upregulation of prolactin mRNA, a pituitary hormone that can antagonize TH action in the tail (Lorenz et al., 2009), but again, no morphological manifestation of this negative interaction between CORT and TH has been measured.

4. Corticosteroid levels across development

The developmental effects of exogenous CORT and ALDO suggest an endogenous role in metamorphosis, and the developmental profiles for CORT (Fig. 1A) and ALDO (Fig. 1B) support this view. In *L. catesbianus*, *L. pipiens*, *Lithobates sylvaticus*, and *B. bufo*, CORT had a single peak at climax (Chambers et al., 2011; Denver, 1998; Glennemeier and Denver, 2002a,b; Jaffe, 1981; Jolivet Jaudet and Leloup Hatey, 1984; Wright et al., 2003). Measurements at earlier premetamorphic stages showed an early level of CORT exceeding a smaller peak at climax in *X. laevis*, but high premetamorphic CORT levels were not observed in *L. pipiens* (Glennemeier and Denver, 2002a). Kloas (1997) found the high premetamorphic CORT levels but not a separate peak at climax in *X. laevis* (Kloas et al., 1997). These hormone profiles showing a large premetamorphic peak in *X. laevis* are not consistent with interrenal histology reflecting low steroidogenic activity in premetamorphosis with increasing degrees of activity as metamorphosis proceeds (Rapola, 1963) No explanation for this discrepancy has been identified.

Peaks in ALDO were observed at metamorphic climax in *L. catesbianus*, *B. bufo*, and *B. japonicus* (Kikuyama et al., 1986; Niinuma et al., 1989; Wright et al., 2003), but in *X. laevis*, ALDO had two peaks, i.e., at prometamorphosis and climax (Jolivet Jaudet and Leloup Hatey, 1984; Kloas et al., 1997). In contrast to the above, Krug et al. found a broad CORT profile across the larval period rather than sharp peak at climax and did not measure an increase in ALDO until after metamorphosis in *L. catesbianus* (Krug et al., 1983). It is possible that these discrepancies may be explained by use of plasma compared to whole body content and time of day and photoperiod (Wright et al., 2003).

Due to variation in interpretation of stages for different species and among researchers, it is difficult to conclude whether the corticosteroid peaks identified among these studies are the same developmental stage or coincide with the stage of the TH peak at metamorphosis (Leloup and Buscaglia, 1977). In any case, most results showed a CORT peak at metamorphic climax at approximately the same stage as the TH peak or a stage or two later, and ALDO had one peak at climax and another during prometamorphosis depending on species.

During a larval stress response, the hypothalamus and pituitary secretions cause release of CORT and TH (Denver, 2013). Numerous stressors have been shown to induce endogenous CORT production, including predation, crowding, starvation, xenobiotic exposure, and water reduction (Belden et al., 2005, 2010; Crespi and Denver, 2005; Denver, 1998; Glennemeier and Denver, 2002a; Maher et al., 2013). No experiment attempted to measure if larval stress treatments also increase ALDO levels. However, ACTH injections stimulated ALDO production in tadpoles and adults of *L. catesbianus* and juveniles of *X. laevis* (Carstensen et al., 1959; Iwamuro et al., 1989; Jaffe, 1981) Consistent with exogenous hormone treatments, the developmental profiles and effects of stress on hormone levels implicate both hormones in developmental action during metamorphosis.

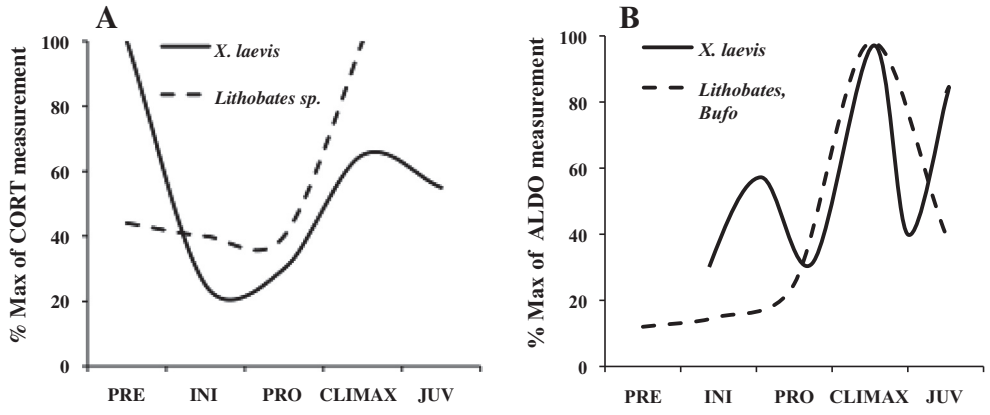

Fig. 1. Consensus corticosteroid developmental profiles. (A) The consensus CORT developmental profile for *Xenopus laevis* shows high premetamorphic levels with a second lower peak at climax (Glennemeier and Denver, 2002a; Jolivet Jaudet and Leloup Hatey, 1984; Kloas et al., 1997), though one study did not observe the second peak at climax (Kloas et al., 1997). The consensus CORT profile for *Lithobates* species (*L. sylvaticus*, *L. catesbianus*, *L. pipiens*) rises to climax, but post-climax levels were not measured (Chambers et al., 2011; Glennemeier and Denver, 2002a; Wright et al., 2003). (B) The consensus ALDO developmental profile for *X. laevis* shows two peaks, one at prometamorphosis and one at climax, though the two studies contrasted on which peak was higher (Jolivet Jaudet and Leloup Hatey, 1984; Kloas et al., 1997). The consensus ALDO profile for *L. catesbianus* showed a single peak at climax (Kikuyama et al., 1986; Wright et al., 2003) but one study on *L. catesbianus* showed no change in ALDO levels until after completion of metamorphosis (Krug et al., 1983). A study on *Bufo japonicus* showed a single peak at the end of climax (Niinuma et al., 1989). Hormone measurements were done by radioimmunoassay on plasma or whole body extracts. PRE: premetamorphosis, INI: initiation of metamorphosis, PRO: prometamorphosis, CLIMAX: climax of metamorphosis, MET: metamorphosis, JUV: juvenile.

5. Corticosteroid receptors

Two types of corticosteroid receptors have been identified in vertebrates, (I) the mineralocorticoid receptor (MR), which binds glucocorticoids and mineralocorticoids with high affinity, and (II) the glucocorticoid receptor (GR), which binds glucocorticoids with a lower affinity than MR and does not bind mineralocorticoids (Sapolsky et al., 2000). Both receptor types were originally cloned 20 years ago in *X. laevis* (Csikos et al., 1995; Gao et al., 1994). MR and GR are found primarily in cytosol in the absence of ligand, bound by several other proteins that maintain the receptors in a conformation favorable to ligand binding (Fig. 2). Upon hormone binding, the cytosolic complex dissociates, and the receptor translocates to the nucleus where it can regulate gene transcription by binding as homodimers to mineralocorticoid or glucocorticoid responsive elements (MRE or GREs) that are present in promoter regions of hormone-responsive genes resulting in increased or decreased gene expression. In addition to the receptors, tissue sensitivity to CSs is regulated by expression of the metabolizing isoenzymes 11β-hydroxysteroid dehydrogenases, which interconvert CSs between active and inactive versions, and multidrug efflux pump, which transports CSs from cells (Quax et al., 2013).

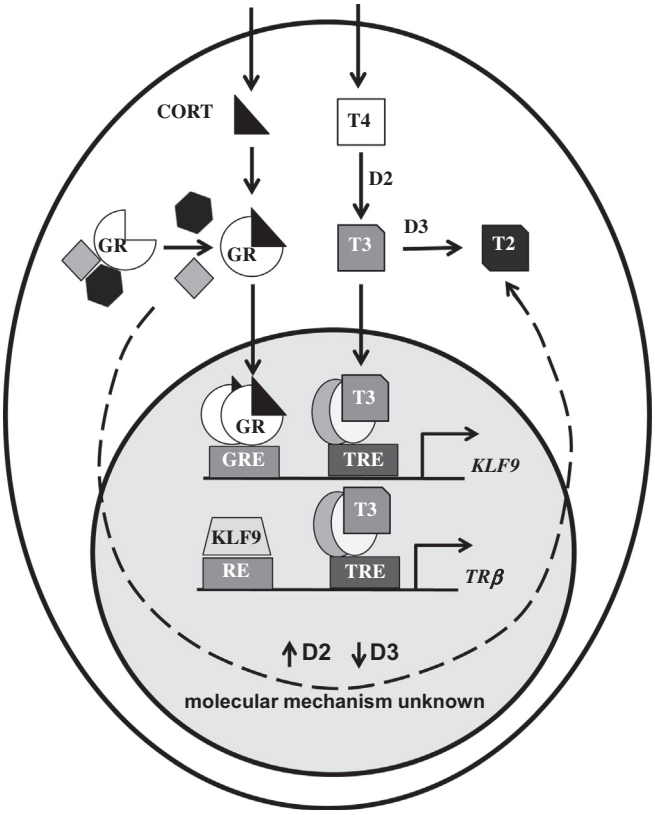

Fig. 2. Diagram of corticosteroid signaling. CORT enters cells from the blood. In the absence of CORT, cytoplasmic proteins retain glucocorticoid receptor (GR) in the cytoplasm and maintain its confirmation favorable to binding by CORT. Once GR is bound by CORT, the cytosolic complex dissociates, and the bound receptor translocates to the nucleus. The known ways CORT affects tissues during metamorphosis are through regulation of transcription of genes whose products modulate TH signaling. The majority of TH enters the cell as T4 and is converted to T3, the active form of TH, by deiodinase type 2 (D2). T3 then enters the nucleus to bind TH receptor and retinoid X receptor heterodimers at TH response elements (TRE) and induce TH-response genes, including Krüpel-like factor 9 (*KLF9*) and TH receptor beta (*TRβ*). Deiodinase type 3 (D3) degrades T4 to reverse T3 (an inactive form, not shown) and T3 to T2 (another inactive form). GR-CORT binds to glucocorticoid response elements (GRE) and induces the expression of *KLF9* in the presence or absence of TH. KLF9 may bind the promoter at a KLF9 response element (RE) and induce *TRβ*. CORT can also increase D2 and decrease D3 mRNA and activity levels by unknown molecular mechanisms, which increase the bioavailability of T3. CORT may bind MR in addition to GR (not shown). Mineralocorticoid gene regulation works like CORT except using ALDO, MR, and MRE. However, the molecular consequences of ALDO-MR gene regulation on TH signaling mechanisms haven not been examined.

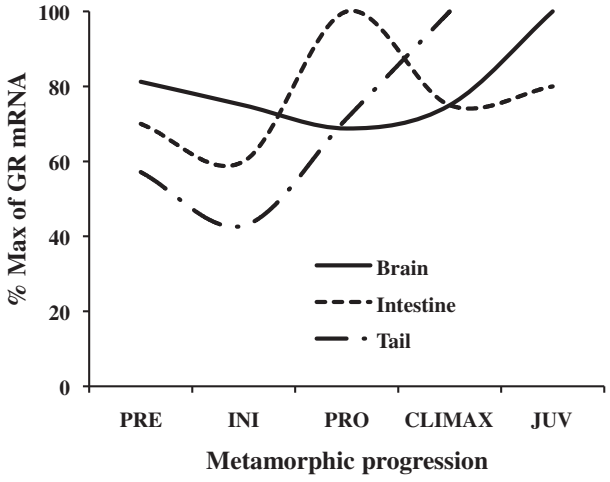

Fig. 3. GR developmental expression profiles. Levels of GR mRNA in brain, intestine, and tail show tissue-specific dynamic expression patterns during metamorphosis in *X. laevis*. Data after Krain and Denver (2004). Abbreviations as in Fig. 1.

GR expression is wide spread among tissues and is hormone regulated. In *X. laevis* and *L. catesbianus*, GR mRNA and protein show dynamic expression changes among stages, hormone treatments, and tissues (brain, liver, intestine, and tail) (Fig. 3) (Bonett et al., 2010; Krain and Denver, 2004; Woody and Jaffe, 1984). GR mRNA levels increased with development in the brain and tail, reaching the highest levels at the completion of metamorphosis, whereas TH treatment repressed GR mRNA in brain and induced it in tail (Bonett et al., 2010; Krain and Denver, 2004). Treatment with CORT had no effect on GR mRNA levels in either tissue. In the intestine, GR mRNA levels remained unchanged throughout development but were reduced after TH-treatment (Krain and Denver, 2004). Cytosolic GR binding capacity showed divergent patterns across development in intestine and tail compared to mRNA perhaps explained by different species and/or measurement of cytosolic rather than whole cell levels (Woody and Jaffe, 1984).

Fewer studies have been conducted on the tissue distribution of MR, but it has been found in many tissues, including outside those typically associated with salt and water balance: (1) skin cytosol contained saturable ALDO binding sites in *Pelophylax esculentus* but not *X. laevis* (Leloup-hatey et al., 1990), (2) MR mRNA was detected in whole bodies of prometamorphic *X. laevis* (Csikos et al., 1995), (3) MR mRNA and protein were present in the pituitary and tail in *X. laevis* and *L. catesbianus* (Bonett et al., 2010; Roubos et al., 2009; Yamamoto and Kikuyama, 1993), and (4) MR mRNA is inducible by TH in the tail of *X. laevis* (Bonett et al., 2010). In XTC-2 cells (unknown tissue origin), both GR and MR were detected, though expression levels were not compared (Bonett et al., 2009). Gene expression analysis using these cells showed that both RU486 (a GR and progesterone receptor antagonist) and spironolactone (an MR antagonist) significantly decreased basal and CORT-induced levels of the CORT-response gene *KLF9*, suggesting the involvement of both GR and MR.

6. Mechanisms of corticosteroid action on metamorphosis

The above studies suggest that both CSs and their receptors are available in many if not most tissues to take part in regulation of

metamorphosis, but the identity of which signaling system carries out which actions endogenously has not been determined. Mechanisms of metamorphic inhibition by CSs in the absence of TH are less well understood than acceleratory effects in the presence of TH. During premetamorphosis, exogenous CORT may block development by feedback inhibition on hypothalamic CRH neurons reducing CRH stimulation of TSH and TH release, as evidenced by reduced plasma concentrations of TH after CORT treatment in *X. laevis* and reduced thyroid follicle cell height and proliferation (Buscaglia et al., 1981; Leloup-hatey et al., 1990; Wright et al., 1994) The growth inhibition due to CORT may indirectly affect metamorphic development by modulating internal energy stores and/or physiology that may signal the brain to reduce production of hypothalamic hormones regulating development (Hu et al., 2008), but little is known about this potential mechanism. Because the above experiments used exogenous CORT and not ALDO, because ALDO has similar inhibitory effects as CORT during premetamorphosis, and because receptor specific agonists and antagonists were not used, it is possible that either or both hormones or receptors could be involved in these inhibitory effects in the absence of TH.

In the presence of TH, mechanisms underlying acceleratory effects of CSs involving central control have not been identified. In the periphery, several mechanisms can account for the acceleratory effects of CSs on TH-induced metamorphosis locally in tissues. First, both ALDO and CORT increased TH-induced nuclear TH binding capacity in cultured tail tips in *B. japonicus* and *L. catesbianus* (Niki et al., 1981; Suzuki and Kikuyama, 1983). Such increased nuclear binding is consistent with CORT-dependent augmentation of TH-induced *TRβ* (TH receptor beta) mRNA expression (Krain and Denver, 2004). Increased tissue sensitivity to TH via increased *TRβ* expression certainly contributes to the synergy of CSs and TH in metamorphosis, and at least two molecular mechanisms may contribute to this action of CSs on *TRβ* and likely other TH-response genes. At the level of TH signaling, CORT can increase the conversion of T4 to T3 (inactive to active version of TH) via deiodinase type 2 (D2) and decrease the degradation of T4 and T3 to inactive metabolites by deiodinase type 3 (D3) (Fig. 2). Specifically, CORT increased D2 activity in skin and decreased D3 activity in liver and gut, consistent with reduced excretion of inactive metabolites and retention of T3 in the plasma (Galton, 1990). In tail, CORT increased D2 and D3 mRNA (Bonett et al., 2010; Lorenz et al., 2009). It is possible that CORT regulates D2 and D3 activity either by direct gene regulation or by indirect regulation of genes that affect deiodinase transcription and/or activity. Direct cross-talk between CORT and TH at the level of gene regulation is exemplified by regulation of *KLF9*, which is induced independently and synergistically by TH and CORT (Bonett et al., 2009). In addition, KLF9 contributes to induction of the TH-response gene *TRβ* in *X. laevis* (Bagamasbad et al., 2008). TR and MR/GR binding sites have been identified in a synergy module upstream of the *KLF9* transcriptional start site, providing a transcriptional mechanism for CORT and TH synergy (Bagamasbad, 2012). These valuable insights into the role of CSs have been gained using exogenous CORT, but whether CORT and/or ALDO or whether GR and/or MR are the endogenous players remains to be determined.

7. Conclusions and future directions

Research on the endocrinology of CSs in frog metamorphosis to understand their roles in vertebrate development began over 70 years ago in experiments showing CORT accelerates TH-induced metamorphosis (Bock, 1938). Since then, many effects of stress or treatment with CSs hormones on tadpoles have been observed. Where examined, CORT, ALDO, and dexamethasone (GR-specific agonist) typically induce similar effects, and metyrapone (corticosteroid synthesis inhibitor), RU486 (GR antagonist), and spironolactone (MR antagonist) have the expected inhibition of corticosteroid actions. However, stress can induce TH as well as CORT (Denver, 1998; Gomez-Mestre et al., 2013), and because exogenous TH is sufficient to recapitulate most metamorphic events *in vivo* and *in vitro* (Dodd and Dodd, 1976), it is not clear the extent to which CORT and/or ALDO affect metamorphic changes. During natural metamorphosis, both CORT and ALDO have peak blood levels at climax, and stress increases circulating CORT, and perhaps ALDO, levels. Also, GR is expressed in all tissues examined, and MR has a broad tissue distribution. Thus, both CSs and their receptors are available to be the endogenous players.

Nevertheless, lack of effective surgical or chemical means to completely and specifically block CORT and ALDO synthesis or action has confounded attempts to define their specific developmental role(s). Dexamethasone has been an underused reagent to illuminate GR-specific effects in tadpoles, but in axolotls, dexamethasone synergizes with TH to accomplish metamorphosis (Kühn et al., 2004) If similar results were found in frogs, then such data in combination with the ability of ALDO to synergize with TH in tadpoles would suggest both GR and MR independently can mediate the corticosteroid developmental signals.

Gene knockout studies in mice and polymorphisms in humans show that birth occurs in the absence glucocorticoid and mineralocorticoid signaling but lethality and failure to thrive occur postnatally. On the other hand, hormonal compensation by the mother have made it difficult to elucidate post-embryonic and fetal developmental roles of CSs. Developmental genetic studies in fish have identified embryonic significance of corticosteroid signaling, but potential developmental roles of mineralocorticoids cannot be addressed in fish. Current gene disruption technologies (TALENs and CRISPRs) can now be used in amphibians to create homozygous mutations in relevant pituitary hormones, steroidogenic enzymes, and hormone receptors to elucidate specific roles of CSs and their receptors in frog metamorphosis.

Another important gap in knowledge of corticosteroid developmental endocrinology is to identify and elucidate roles of downstream target genes of CSs alone and in cross-talk with TH. Though CORT and TH synergy as well as antagonism have been identified as major classes of gene regulation patterns, effects of corticosteroid-regulated genes have not been experimentally examined nor have the molecular mechanisms of their regulation been elucidated. Such studies require gene expression analysis and/or manipulation of expression levels using transgenic/knockout techniques. Elucidating the molecular mechanisms using mutant receptors and chromatin immunoprecipitation of such CORT-induced gene regulation and cross-talk between TH and CSs will require knowledge of the endogenous players in corticosteroid signaling. In conclusion, many endocrine actions of exogenous CSs have been identified, but knowledge of the actual endogenous hormone and mediating nuclear receptor, upstream mechanisms of CS tissue-sensitivity, and downstream consequences of CS action have not been determined in most cases but are required to understand the role of CSs in development in an environmental context.

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