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Molecular mechanisms of corticosteroid synergy with thyroid hormone during tadpole metamorphosis

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ABSTRACT

Corticosteroids (CS) act synergistically with thyroid hormone (TH) to accelerate amphibian metamorphosis. Earlier studies showed that CS increase nuclear 3,5,3′-triiodothyronine (T_3) binding capacity in tadpole tail, and 5′ deiodinase activity in tadpole tissues, increasing the generation of T_3 from thyroxine (T_4). In the present study we investigated CS synergy with TH by analyzing expression of key genes involved in TH and CS signaling using tadpole tail explant cultures, prometamorphic tadpoles, and frog tissue culture cells (XTC-2 and XLT-15). Treatment of tail explants with T_3 at 100 nM, but not at 10 nM caused tail regression. Corticosterone (CORT) at three doses (100, 500 and 3400 nM) had no effect or increased tail size. T_3 at 10 nM plus CORT caused tails to regress similar to 100 nM T_3. Thyroid hormone receptor beta (TRβ) mRNA was synergistically upregulated by T_3 plus CORT in tail explants, tail and brain *in vivo*, and tissue culture cells. The activating 5′ deiodinase type 2 (D2) mRNA was induced by T_3 and CORT in tail explants and tail *in vivo*. Thyroid hormone increased expression of glucocorticoid (GR) and mineralocorticoid receptor (MR) mRNAs. Our findings support that the synergistic actions of TH and CS in metamorphosis occur at the level of expression of genes for TRβ and D2, enhancing tissue sensitivity to TH. Concurrently, TH enhances tissue sensitivity to CS by upregulating GR and MR. Environmental stressors can modulate the timing of tadpole metamorphosis in part by CS enhancing the response of tadpole tissues to the actions of TH.

1. Introduction

Amphibian metamorphosis is dependent on thyroid hormone (TH), which induces the suite of molecular and cellular changes that cause a tadpole to transform into a frog. Thyroid hormone actions are mediated by TH receptors (TRs, TRα and TRβ) that are members of the nuclear receptor (NR) superfamily and function as ligand-activated transcription factors (Mangelsdorf et al., 1995). In *Xenopus laevis*, TRα is expressed shortly after hatching and is maintained at a relatively constant level throughout tadpole life and metamorphosis (reviewed by Shi, 2000). TRα may establish tissue competence to respond to TH once the thyroid gland matures and starts to secrete hormone, and may mediate the actions of TH on cell proliferation (Denver et al., 2009; Furlow and Neff, 2006). By contrast, TRβ is expressed at a low level throughout tadpole life, and increases dramatically at the onset of metamorphosis when the plasma TH concentration rises (Shi, 2000). The expression of TRβ is dependent on the rise in plasma TH; i.e., it is autoinduced, and the level of TRβ expression in target cells is predicted to play a central role in determining the responsiveness of the cell to TH (reviewed by Furlow and Neff, 2006; Tata, 2006).

In addition to the amount of TR made by a cell, an important determinant of TH action during metamorphosis is the activity of intracellular enzymes (deiodinases) that establish the concentration of bioactive TH within the cell (St Germain et al., 2009). The major product of the amphibian thyroid gland is thyroxine (T_4) with minor amounts of 3,5,3′-triiodothyronine (T_3) produced (Buscaglia et al., 1985; Rosenkilde, 1978). The biologically active form of TH is T_3, which has up to ten times greater activity than T_4 (Frieden, 1981; Lindsay et al., 1967; Rosenkilde, 1978; Wahlborg et al., 1964; White and Nicoll, 1981). Of the three deiodinases expressed in amphibian tissues, two appear to play dominant roles during metamorphosis: the 5′ deiodinase type 2 (D2), which converts T_4 to T_3, and the 5 deiodinase type 3 (D3), which converts T_4 to reverse T_3 (3,3′,5′-triiodothyronine; rT_3), and T_3 to 3,5-diiodo-L-thyronine (T_2), both of which are inactive in that they do not bind to TRs (reviewed by Denver, 2009a). The balance of D2 and D3 activity within a cell is hypothesized to establish the intracellular level

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of bioactive hormone, although precisely how this balance is achieved is not well understood (St Germain et al., 2009).

Hormones other than TH play important roles in amphibian metamorphosis, in part by modifying the production and actions of TH. For example, pituitary prolactin may reduce tissue responsiveness to TH by blocking TRβ autoinduction. This action of PRL may be important during metamorphic climax when PRL expression increases, possibly to control the morphogenic actions of TH (reviewed by Denver, 2009a). Corticosteroids (CS), hormones produced by adrenocortical cells (interrenal glands in frogs), synergize with TH at target tissues to promote morphogenesis (Denver, 2009b; Kikuyama et al., 1993). The production of CS changes with development, rising throughout metamorphosis and reaching a peak at metamorphic climax (Kikuyama et al., 1993). Also, because CS are stress hormones, their production is increased by exposure to environmental stressors such as habitat dessication, competition for resources, predation risk, etc. (Denver, 2009b). Various environmental stressors have been shown to accelerate tadpole metamorphosis, and this developmental acceleration may be mediated by stress hormones produced both centrally (central nervous system – CNS and pituitary gland) and peripherally (interrenal glands; Denver, 2009b). At the level of the CNS and pituitary gland, hypothalamic corticotropin-releasing factor (CRF) has a dual hypophysiotropic role in tadpoles, stimulating the secretion of pituitary adrenocorticotropic hormone (ACTH), which increases CS production by adrenocortical cells, and thyroid stimulating hormone (TSH), which increases hormone secretion by the thyroid gland. At the level of hormone target cells in peripheral tissues, CS have been shown to synergize with TH to promote morphogenesis (Denver, 2009b; Kikuyama et al., 1993).

Like TH, CS actions are mediated by NRs encoded by two different genes: the type I (mineralocorticoid receptor; MR) and the type II (glucocorticoid receptor; GR). Two mechanisms have been proposed to explain CS synergy with TH during tadpole metamorphosis. Work from Kikuyama's group showed that CS increase nuclear T_3 binding capacity in tadpole tail (reviewed by Kikuyama et al., 1993). Galton (1990) found that CS influence deiodinase activity in tadpole tissues, increasing 5′ deiodinase and decreasing 5 deiodinase. These findings suggest that CS act to enhance cellular responsiveness by upregulating TRs and at the same time increasing conversion of the precursor T_4 to the biologically active hormone T_3. Thus, stress hormones mediate environmental effects on development, and they interact with the thyroid axis at both central and peripheral levels.

In the current study we examined morphological and gene expression changes in tadpole tissues and cell lines caused by TH, CS, or combined treatment with the two hormones. We investigated whether the synergy between TH and CS occurs at physiological doses of the hormones, and is seen at the level of TR and deiodinase gene expression. We also investigated the expression of CS receptors, to determine if they are autoregulated, or crossregulated by TH. Our findings provide a molecular basis for understanding the enhanced sensitivity of tadpole tissues to TH caused by increased CS, and support a mechanism for accelerated metamorphosis via interactions among the thyroid and stress hormone axes at the hormone target tissues.

2. Materials and methods

2.1. Animal care

We raised *Xenopus laevis* and *Silurana tropicalis* tadpoles obtained from in-house breeding in dechlorinated tap water (water temperature 21–23 °C – *X. laevis*; 24–25 °C – *S. tropicalis*) under a 12L:12D photoperiod and fed frog brittle (*X. laevis*; NASCO, Fort Atkinson, WI) or Seramicron (Sera North America, Inc., Montgomeryville, PA) plus boiled spinach (*S. tropicalis*). All procedures involving animals were conducted in accordance with the guidelines of the University Committee on the Care and Use of Animals of the University of Michigan.

2.2. Tissue explant culture and hormone treatment

We initiated tadpole tail explant cultures to investigate the actions of T_3 and corticosterone (CORT) on tail regression and gene expression. We treated Nieuwkoop–Faber stage 52–54 (Nieuwkoop and Faber, 1956) *X. laevis* tadpoles with oxytetracycline (100 μg/ml in aquarium water) for 24 h prior to dissection. Dissections were carried out under semi-sterile conditions. Tadpoles were anesthetized in 0.01% benzocaine and dipped in 70% ethanol to sterilize the epidermis. The tails were dissected into sterile 6-well tissue culture dishes containing 2 ml ice-cold tissue culture medium ($n = 7$/treatment for morphological measurements, $n = 6$/treatment for RNA analyses). Tail explants were cultured in Dulbeco's Modified Eagle's Medium (DMEM; Gibco BRL) diluted 1:1.5 for amphibian tissues at 25 °C in an atmosphere of 5% CO_2 and 95% O_2 with gentle rotation (50 rpm). The T_3 was dissolved in 0.01 N NaOH and CORT was dissolved in 100% ethanol. All treatments received an equivalent amount of ethanol vehicle (0.001%). Tail explants were treated with T_3 (10 or 100 nM), CORT (100, 500 or 3400 nM) or 10 nM T_3 plus CORT (100, 500 or 3400 nM). The medium was replaced every 12 h. For RNA analysis, three tails were harvested per treatment 2 days after initiation of hormone treatments; the remaining three tails per treatment were harvested at 5 days. Tails were rinsed twice in ice-cold Dulbecco's Phosphate-Buffered Saline (DPBS; Sigma Chemical Co., St. Louis, MO), snap frozen in a dry ice-ethanol bath and stored at −80 °C until RNA extraction.

We conducted morphometric analysis of tail explants by measuring tail area and final dry weight. For tail area, we captured images every day for seven days with a digital camera and then used Scion Image software (v. 3.0 Scion Corporation, Frederick, MD, USA) to trace the perimeter of each tail and calculate the area. At the end of the experiment we dried the tails in a drying oven and recorded the final dry weight.

2.3. Treatment of tadpoles with hormones in vivo

To investigate gene expression responses to T_3 and CORT *in vivo* we treated NF stage 52 *S. tropicalis* tadpoles with T_3 and CORT for 24 h before sacrifice. We maintained tadpoles in 4 L aquaria (6 tadpoles per aquarium) and hormones were added to the aquarium water. All treatments received an equivalent amount of ethanol vehicle (0.001%). Tadpoles were treated with T_3 (1, 5 or 50 nM), CORT (100 nM) or 100 nM CORT plus T_3 (1 or 5 nM) and the aquarium water was changed and hormones replenished at 12 h. At sacrifice tail and brain (a region encompassing the preoptic area/diencephalon) were harvested, snap frozen in a dry ice-ethanol bath and stored at −80 °C for subsequent RNA isolation.

2.4. X. laevis tissue culture cells

To further investigate gene expression responses to T_3 and CORT, and to establish a cell culture model system for future detailed molecular analyses of hormone synergy we treated the *X. laevis* embryonic fibroblast-derived cell line XTC-2 and the tadpole tail myoblast-derived cell line XLT-15 with hormones and measured gene expression by reverse transcriptase-polymerase chain reaction (RT-PCR). We cultured cells in Leibovitz L-15 medium (diluted 1:1.5 for amphibian tissues) plus 10% Fetal Bovine Serum (Invitrogen, Carlsbad, CA; growth medium) that was stripped of

TH and steroids (Samuels et al., 1979; Yao et al., 2008) at 25 °C in an atmosphere of 5% CO_2. All cell culture experiments were performed in 6-well plates. We plated cells overnight in growth medium at a density of 2×10^5 cells/well (XTC-2) and 5×10^4 cells/well (XLT-15). We replaced the growth medium with serum-free L-15 and continued the cultures for 24 h before adding hormones. Cells reached approximately 75% confluency at the start of hormone treatments. We treated cells with hormones or vehicle (ethanol; 0.001%) for 4 h (XTC-2) or 14 and 24 h (XLT-15). At the end of the experiment we rinsed cells twice with DPBS then harvested them into 1 ml Trizol (Invitrogen) and proceeded with RNA extraction (described below).

2.5. RNA isolation and gene expression analyses

We isolated total RNA from tail explants, freshly dissected tadpole tissues or cell lines using Trizol reagent (Invitrogen) following the manufacturer's instructions. We analyzed gene expression by either Northern blotting (tail explants), semi-quantitative RT-PCR (XLT-15 cells) or quantitative real time RT-PCR (RTqPCR; tadpole tail and brain, XTC-2 cells) using Taqman assays.

We prepared Northern blots by electrophoresing 10 μg of total tail RNA per lane in a 1% agarose–formaldehyde gel and transferring the RNAs to nylon membranes. Blots were prehybridized in Ultrahyb (Ambion) for 30 min and hybridized for 16 h at 42 °C with cDNAs labeled with [^{32}P]-dCTP by random priming (Amersham Corp.). Blots were then washed with 2× saline sodium citrate (SSC) buffer (0.3 M NaCl, 30 mM trisodium citrate, pH 7.0), 0.5% SDS at room temperature for 10 min and then with 0.25× SSC, 0.1% SDS at 65 °C for 1 h before exposure to X-ray film for 1–14 days. Blots were consecutively stripped and reprobed with [^{32}P]-labeled cDNA probes for *X. laevis* genes. The genes analyzed were TRA1 (Genbank Accession # M35343), TRβA1 (M35359), GR (X72211), MR (U15133), D2 (AF354707) and D3 (L28111). To normalize for RNA loading blots were probed for ribosomal protein L8 (rpL8; U00920). X-ray images were digitally captured and densitometric quantitation of the mRNA expression was done using Scion Image software.

For semi-quantitative RT-PCR we treated 1 μg total RNA with 1.5 U RNase-free DNase I (Roche, Indianapolis, IN, USA) to digest contaminating genomic DNA, then reverse-transcribed the RNA into cDNA using 250 ng random hexamers and Superscript II reverse transcriptase following the manufacturer's instructions (see also Bagamasbad et al., 2008; Manzon and Denver, 2004). Minus RT controls were included to verify no genomic DNA contamination. For PCR we used HotStar Taq DNA polymerase following the manufacturer's instructions (Qiagen, Valencia, CA, USA). Deoxyoligonucleotide primer sequences used for PCR are given in Table 1. Reaction volumes were 50 μl, and the reaction included an initial 15 min denaturation and activation step at 95 °C. The PCR cycling conditions were 94 °C for 45 s, 57–63 °C for 45 s, and 72 °C for 60 s. The number of cycles used for each gene was determined empirically using a linear amplification range (Table 1). PCR products were electrophoresed in a 1.5% agarose gel and the bands stained with ethidium bromide. Stained bands were captured digitally using a closed-circuit television camera and densitometric analyses were conducted using Scion Image software.

For quantitative real time RT-PCR (RTqPCR) we synthesized cDNAs from 1 μg DNase-treated total RNA as described above. Custom Taqman assays (Applied Biosystems Inc.) were designed for TRα, TRβ, GR, MR, D2, D3, and rpL8 for *X. laevis* and *S. tropicalis* (genome version 4.1; Table 1). Quantitative PCR was conducted using ABsoluteTM qPCR low Rox Mix (ABgene) and reactions were run on either an ABI 3500 or an ABI StepONE Plus qPCR machine. Relative quantities were determined using standard curves generated with pooled cDNAs.

2.6. Data analysis and statistics

We normalized all gene expression data to the level of expression of the housekeeping gene rpL8. Northern blot data shown on the graphs are expressed as a percentage of maximum expression normalized to rpL8, but statistics were calculated from band densities for each gene normalized to rpL8. Semi-quantitative and quantitative RT-PCR data are given as ratios of gene expression to rpL8; the normalized gene expression data were $\log_{10}$-transformed before statistical analysis. We analyzed morphometric and gene expression data by one-way analysis of variance (ANOVA) using SYSTAT statistical software (v. 10). Fisher's least significant difference (Fisher's LSD) test was used to test for significant differences among individual treatments ($\alpha = 0.05$). We define synergy between T_3 and CORT as: (1) no effect of either hormone alone at the dose tested, but a significant effect with combined treatment, or (2) an effect of combined hormone treatment that is greater than the estimated additive effect by one standard deviation of the combined hormone treatment mean.

3. Results

3.1. Corticosterone synergizes with T_3 to cause tadpole tail regression in vitro

Several groups have reported regression of tadpole tail explants by treatment with TH or TH plus CS (Gray and Janssens, 1990; Kikuyama et al., 1993). Here, we first report confirmation of these findings as a basis for investigating hormone-dependent gene expression changes using this experimental paradigm. We analyzed the effects of hormone treatments on tail area daily over a seven day period, and tail dry weight at the end of the experiment. We treated tails from NF stage 52–54 tadpoles with a dose of T_3 that was previously found not to affect tail regression over a 7 day period (10 nM) and a dose that caused significant tail regression as a positive control (100 nM; Gray and Janssens, 1990; Robinson et al., 1977). We chose three doses of CORT for the experiment: 100, 500 and 3400 nM. The high dose, 3400 nM, which would generate supraphysiological tissue content of CORT, was used previously by Gray and Janssens (1990), and we chose this dose in an attempt to replicate their findings. We also chose lower doses of CORT, 100 and 500 nM, to generate changes in tissue CORT content within the physiological range for tadpoles. We chose the 100 nM dose because it elevated whole body CORT within the physiological range in tadpoles, and also caused phenotypic and gene expression responses in tadpoles, frogs and frog cells (Bonett et al., 2009; Glennemeier and Denver, 2002; Hu et al., 2008; Yao et al., 2008). We chose the 500 nM dose to generate tissue CORT content in the high physiological range that is achieved during a stress response (Krain and Denver, 2004; Yao et al., 2008).

We saw a significant effect of treatment ($F_{(8, 413)} = 43.919$, $P < 0.0001$) and day ($F_{(6, 413)} = 32.918$, $P < 0.0001$) on tail area (Fig. 1A), and a significant interaction between treatment and time ($F_{(48, 413)} = 3.795$, $P < 0.0001$). Tail area diverged among treatments by day 4 ($F_{(8, 60)} = 5.194$, $P < 0.0001$). At day 7, the areas of tails treated with 100 nM T_3, or T_3 plus all three doses of CORT were not different from each other, but all were significantly smaller than the control, CORT only treatments, or 10 nM T_3 ($F_{(8, 55)} = 31.833$, $P < 0.0001$). The final dry weight of tails treated with 100 nM T_3, or T_3+ CORT (all doses) was significantly less than the control, CORT only, or 10 nM T_3 treatments (Fig. 1B; $F_{(8, 54)} = 9.699$, $P < 0.0001$). Treatment with CORT alone tended to increase final tail dry weight, and this was significantly different from controls for the 3400 nM dose.

Table 1
Primers and probes used for semi-quantitative and quantitative real time RT-PCR analysis of gene expression in frog tissues and cells.

Gene	Primer (sense above, antisense below; 5′ → 3′)	PCR product (bp)	Annealing temp (°C)	# cycles	GenBank Accession #
Primers for semi-quantitative RT-PCR – X. laevis genes					
TRβA	GGCAACAGACTTGGTTTTGG AAGTCCACTTTTCCACCCTC	275	57	36	M35359
D2	AGCCATAAATAGCCTGCCCTGTG GGAACCTTAACTTTGGGACTGC	278	57	34	AF354707
GR	TCA CCT GGG ACT AGC ATT GCA GAA AGC TGC CAC AAG TCA GTA CTC CAT	256	59.9	34	X72211
rpL8	CACAGAAAGGGTGCTGCTAAG CAGGATGGGTTTGTCAATACG	477	63	34	U00920

Gene	Primer (sense above, antisense below; 5′ → 3′)	Taqman probe
Primers and probes for quantitative RT-PCR – X. laevis genes		
TRαA	GGGCAGCCACTGGAAACA GGGAGACTGCCCGATATCTTC	CGTAGGAAGTTTCTGCC
TRβA	GGAAGCCACTGGAAACAGAAAA CATTAACTATGGGAGCTTGTCCAA	AAAATTTTTGCCAGAGGAC
D2	AAGGGCTGCGCTGTGTGT GTCCCAGCTTAACCTGCTTGTAG	AACAGCTTCCTATTGGATG
D3	ACCGCTGGTTGTCAATTTCG TGCAGGCGAGCCATGAA	CTGCACCTGACCCC
GR	CTGGCAGCGCTTTTACCAA ATTCTCAGCCACCTCATGCAT	TGACAAAGCTATTGGACTC
MR	ATGGTTCAGGTGGTGAAATGG GGTCCTCCAGAGGCAAATTTCT	CAAAAGTGATTCCAGGATT
rpL8	TTTGCTGAAAGAAATGGCTACATC CACGGCCTGGATCATGGA	AGGGTATTGTGAAAGACA
Primers and probes for quantitative RT-PCR – S. tropicalis genes		
TRα	GGGCAGCCACTGGAAACA GGGAGACTGCCCGATATCTTC	CGTAGGAAGTTTCTGCC
TRβ	GGAAGCCACTGGAAACAGAAAA CATTAACTATGGGAGCTTGTCCAA	AAAATTTTTGCCAGAGGAC
D2	AAGGGCTGCGCTGTGTGT GTCCCAGCTTAACCTGCTTGTAG	AACAGCTTCCTATTGGATG
D3	ACCGCTGGTTGTCAATTTCG TGCAGGCGAGCCATGAA	CTGCACCTGACCCC
GR	CTGGCAGCGTTTTTATCAA ATTTTCAGCCACCTCATGCAT	TGACAAAGCTATTGGACTC
MR	ATGGTTCAGGTGGTGAAATGG GGTCCTCTAGAGGCAAATTCCT	CAAAAGTGATTCCAGGATT
rpL8	TTTGCTGAAAGAAATGGCTACATC CACGGCCTGGATCATGGA	AGGGTATTGTGAAAGACA

3.2. Corticosterone synergizes with a low dose of T_3 to induce gene expression in tadpole tail explants

Hormone treatments caused significant elevations in TRβ mRNA in tadpole tail explants (48 h $F_{(8,\ 17)} = 4.854$, $P < 0.005$; 120 h $F_{(8,\ 17)} = 5.103$, $P < 0.004$). Thyroid hormone receptor β mRNA was autoinduced, a well known phenomenon that occurs in tadpole tissues during metamorphosis (Fig. 2; reviewed by Tata, 2006). Treatment with CORT alone elevated TRβ mRNA at 48 h but not at 120 h. Corticosterone plus 10 nM T_3 elevated TRβ mRNA to a level that was greater than additive at all doses and timepoints analyzed. The effects of the hormones were synergistic (synergy as defined earlier – see Section 2.6) at 48 h for the 100 nM CORT dose, and at 120 h for the 500 and 3400 nM CORT doses. Although there were trends in TRα mRNA levels caused by hormone treatments that were similar to those observed for TRβ, these changes were not statistically significant (data not shown).

Deiodinase type 2 mRNA in control tail explants was undetectable in our assay (even after 2 week exposure of the Northern blots), which agrees with the findings of Shintani et al. (2002). Because the controls were undetectable we could not conduct a comparative densitometric analysis. Representative Northern blots are shown in Fig. 3. The 100 nM dose of T_3 caused a small increase in D2 mRNA at 48 h, and this appeared greater at 120 h. Neither the 10 nM dose of T_3 nor any of the doses of CORT alone affected D2 mRNA at any time point. Combined treatment with 10 nM T_3 plus CORT at each of the doses tested increased D2 mRNA, and this effect appeared greater at the 120 h time point. Thus, combined treatment with a low dose of T_3 plus CORT caused synergistic upregulation of D2 mRNA.

Deiodinase type 3 mRNA was detectable in control tail explants, similar to what was found previously in tadpole tail by Brown et al. (1996). Only the 100 nM dose of T_3 caused a statistically significant ($P < 0.05$) increase that was comparable in magnitude at both time points measured (data not shown). Treatment with CORT with or without T_3 tended to reduce D3 mRNA in tail explants at both time points but these effects were not statistically significant. GR mRNA was upregulated by 100 nM T_3 at both time points ($P < 0.05$) but unaffected by CORT alone or in combination with T_3 (data not shown; see also Krain and Denver, 2004). Although we detected MR mRNA in tail by Northern blotting we were unable to quantify its expression.

3.3. Corticosterone synergizes with T_3 to induce gene expression in tadpole tail in vivo

We treated NF stage 52 *S. tropicalis* tadpoles with hormones (1, 5 or 50 nM T_3, 100 nM CORT, 1 nM T_3 + 100 nM CORT, 5 nM T_3 + 100 nM CORT) by addition to the aquarium water for 24 h before sacrifice and tissue harvest. Hormone treatments caused sig-

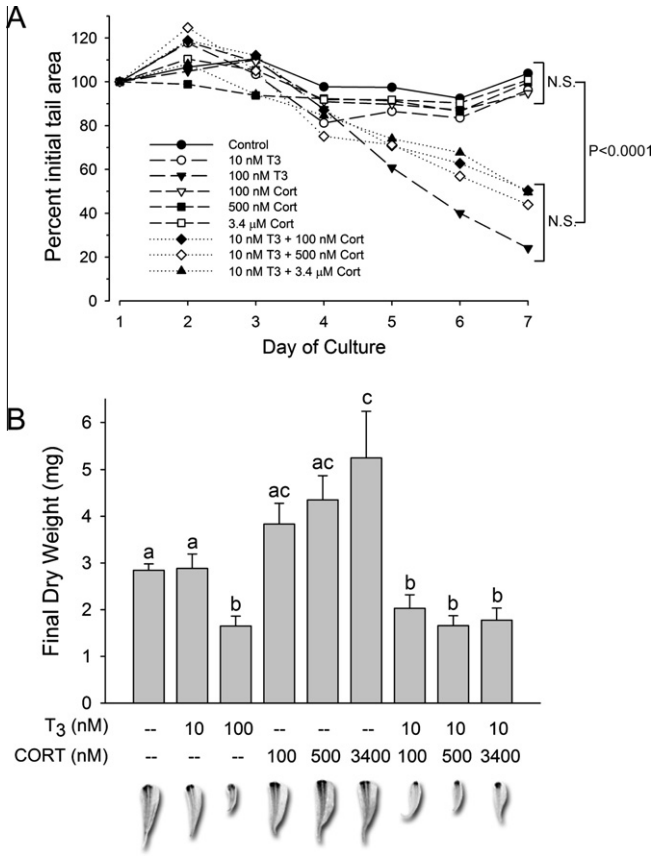

Fig. 1. Corticosterone (CORT) synergizes with 3,5,3′-triiodothyronine (T_3) to cause regression of tadpole tail explants. Tails were harvested from NF stage 52–54 *X. laevis* tadpoles and cultured for 1 week (see Section 2). The medium was replenished and hormones were added at the indicated doses every 12 h. Tails were imaged and area calculated daily, and tails were dried in an oven and final dry weight measured at the end of the experiment. (A) Percent initial tail area over the 7 day culture period. Statistical analyses were based on the actual tail areas (ANOVA; $n = 7$/treatment). (B) Final dry weight of tadpole tail explants. Bars represent means + SEM. Data were analyzed by one-way ANOVA and letters indicate significant differences between the group means based on Fisher's LSD ($P < 0.05$; $n = 7$/treatment). Representative images of tails from each treatment group at the 7 day time point are shown below the graphs.

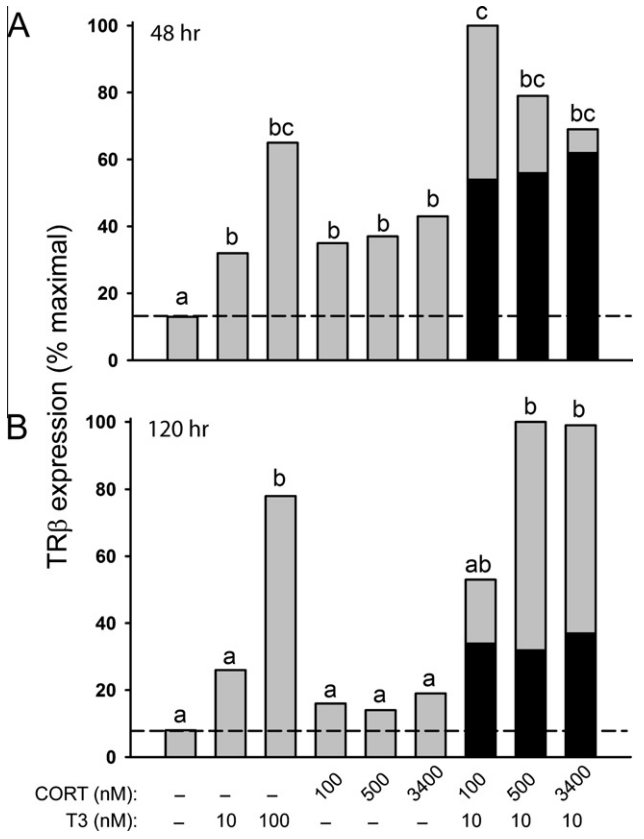

Fig. 2. Corticosterone (CORT) synergizes with 3,5,3′-triiodothyronine (T_3) to induce thyroid hormone receptor beta (TRβ) mRNA in tadpole tail explants. Tails were harvested from NF stage 52–54 *X. laevis* tadpoles and cultured for 1 week (see Section 2). The medium was replenished and hormones were added at the indicated doses every 12 h. Three tails were harvested from each group at 48 h (A) and at 120 h (B) after initiation of hormone treatments and analyzed for TRβ mRNA levels by Northern blotting and densitometry of autoradiograms. The data shown in the graph are the percent of maximal expression, but statistical analyses were calculated from band densities for each gene normalized to rpL8. The data were first $\log_{10}$-transformed before analysis by ANOVA, and letters indicate significant differences between the group means based on Fisher's LSD ($P < 0.05$). The dashed lines indicate the control level of expression. The black bars represent the estimated additive responses to T_3 or CORT alone at the doses indicated.

nificant changes compared with controls in expression of all genes analyzed in the tadpole tail: TRα ($F_{(6, 35)} = 11.1$, $P = 0.0001$), TRβ ($F_{(6, 35)} = 38.22$, $P < 0.0001$), GR ($F_{(6, 35)} = 10.6$, $P < 0.0001$), MR ($F_{(6, 35)} = 3.2$, $P < 0.012$), D2 ($F_{(6, 29)} = 13.07$, $P < 0.0001$) and D3 ($F_{(6, 35)} = 6.4$, $P < 0.0001$). TRα mRNA was increased by T_3 (5 and 50 nM) or CORT (100 nM) alone, and the increase was greater than additive for the combined hormone treatments; it was synergistic for the 5 nM dose of T_3 (Fig. 4A). TRβ mRNA was increased by all doses of T_3 tested, but not by CORT alone. The increase in TRβ mRNA was synergistic for the 5 nM dose of T_3 plus CORT (Fig. 4B).

Deiodinase type 2 mRNA was increased by treatment with 50 nM T_3 or 100 nM CORT (Fig. 5A). Combined treatment with 100 nM CORT plus 1 or 5 nM T_3 also caused significant increases in D2 mRNA, but these actions were not additive nor synergistic. Like D2, D3 mRNA was also increased by 50 nM T_3 or 100 nM CORT (Fig. 5B). Combined treatment with 100 nM CORT plus 1 or 5 nM T_3 produced responses that were greater than additive, and this effect was synergistic for the 5 nM T_3 dose.

Glucocorticoid receptor mRNA was increased by 50 nM T_3, but was not affected by the lower doses of T_3 or by CORT, nor by any of the combined hormone treatments (Fig. 6A). Similar to GR, MR mRNA was increased by 50 nM T_3, but not by 1 nM T_3 or by CORT; there was a trend towards increased MR mRNA by 5 nM T_3 and the combined treatment of 5 nM T_3 plus CORT (Fig. 6B).

In the same experiment we analyzed TRβ and D2 mRNA expression in the brain (preoptic area/diencephalon; Supplemental Fig. S1). Hormone treatments caused statistically significant changes in TRβ mRNA ($F_{(6, 35)} = 62.24$, $P < 0.0001$); TRβ was strongly autoinduced by all doses of T_3 tested (Fig. S1A). Treatment with CORT caused a small but statistically significant increase in TRβ mRNA. Combined treatment of 5 nM T_3 plus CORT caused synergistic upregulation of TRβ mRNA. Hormone treatments caused statistically significant changes in D2 mRNA ($F_{(6, 35)} = 4.94$, $P = 0.001$). D2 was increased by 50 nM T_3, but decreased by 100 nM CORT; other hormone treatments had no statistically significant effects (Fig. S1B).

3.4. Corticosterone and T_3 influence gene expression in X. laevis tissue culture cells

The embryonic fibroblast-derived *X. laevis* cell line XTC-2 responds to T_3 in a dose and time-dependent manner by strongly upregulating TRβ mRNA (Iwamuro and Tata, 1995; Kanamori and Brown, 1992) and it expresses both GR and MR (Bonett et al., 2009). We therefore tested for effects of CORT with or without

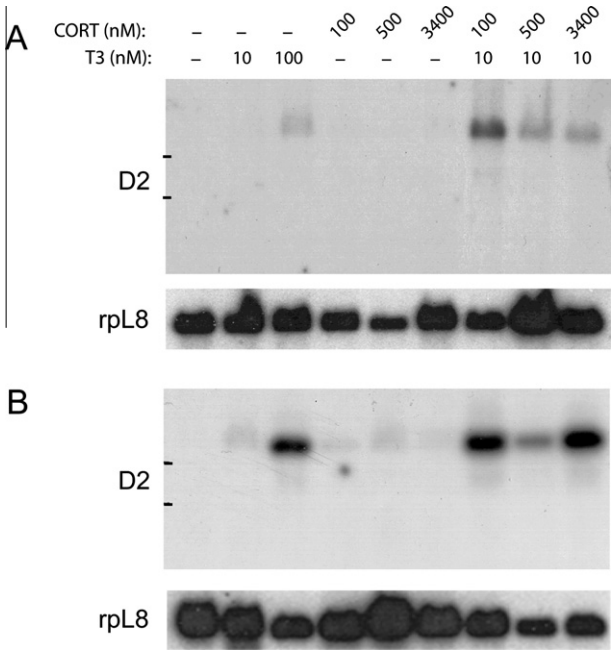

Fig. 3. Corticosterone (CORT) synergizes with 3,5,3′-triiodothyronine (T_3) to induce deiodinase type 2 (D2) mRNA in tadpole tail explants. Tails were harvested from NF stage 52–54 *X. laevis* tadpoles and cultured for 1 week (see Section 2). The medium was replenished and hormones were added at the indicated doses every 12 h. Three tails were harvested from each group at 48 h (A) and at 120 h (B) after initiation of hormone treatments and analyzed for D2 mRNA levels by Northern blotting and densitometry of autoradiograms. Shown are representative Northern blots for both time points. Densitometry could not be conducted because the controls were undetectable.

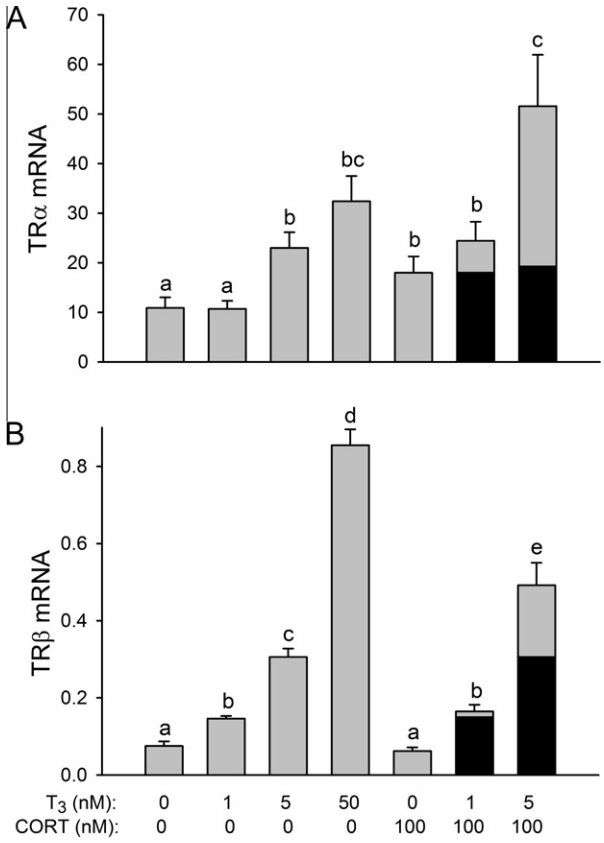

Fig. 4. Corticosterone (CORT) synergizes with 3,5,3′-triiodothyronine (T_3) to induce thyroid hormone receptor alpha (TRα; A) and TRβ (B) mRNAs in tail from tadpoles treated with hormones *in vivo*. Nieuwkoop–Faber stage 52 *S. tropicalis* tadpoles were treated with T_3 and CORT at the doses indicated for 24 h before sacrifice as described in Section 2. Gene expression was analyzed by RTqPCR using Taqman assays. The data are given as ratios of gene expression to rpL8; the normalized gene expression data were $\log_{10}$-transformed before statistical analysis using ANOVA. The letters indicate significant differences between the group means based on Fisher's LSD ($P < 0.05$; $n = 5$/treatment). The black bars represent the estimated additive responses to T_3 or CORT alone at the doses indicated.

T_3 on gene expression in XTC-2 cells. We treated cells with 1 nM T_3 or 1 nM T_3 + 100 nM CORT for 4 h to analyze early gene expression responses. Hormone treatments caused statistically significant changes in expression of mRNAs for TRα ($F_{(3, 17)} = 16.12$, $P < 0.0001$), TRβ ($F_{(3, 20)} = 139.335$, $P < 0.0001$), D3 ($F_{(3, 17)} = 224.29$, $P < 0.0001$), GR ($F_{(3, 16)} = 25.35$, $P < 0.0001$) and MR ($F_{(3, 17)} = 125.32$, $P < 0.0001$) but not for D2 (Fig. 7). TRα mRNA was increased by treatment with T_3 or CORT, but no synergistic action was observed. TRβ mRNA was increased by T_3 but not by CORT; the response of TRβ mRNA to T_3 was considerably greater than that of TRα (TRβ > 130-fold; TRα 1.5-fold). Combined treatment of T_3 with CORT caused synergistic upregulation of TRβ mRNA (Fig. 7).

The expression of D3 mRNA was strongly increased by 1 nM T_3, and although CORT alone had no effect, CORT reduced the T_3-dependent increase by one half. GR mRNA was reduced by CORT, but induced by T_3. Cotreatment with CORT blocked the T_3-induced increase in GR mRNA. The expression of MR mRNA was strongly induced by T_3; CORT had no effect alone or in combination with T_3.

We also investigated the effects of T_3 and CORT on TRβ and GR mRNA expression in the tadpole tail myoblast-derived cell line XLT-15 (Fig. 8). We found statistically significant effects of hormone treatments on TRβ (14 h: $F_{(4, 25)} = 31.44$, $P < 0.0001$; 24 h: $F_{(4, 25)} = 24.26$, $P < 0.0001$) and GR mRNA ($F_{(4, 25)} = 3.74$, $P = 0.016$; $F_{(4, 25)} = 7.18$, $P = 0.001$). Expression of TRβ mRNA was increased by both doses of T_3 at each of the two time points investigated (Fig. 8A). Corticosterone alone had no effect, but CORT synergized with T_3 to increase TRβ mRNA. GR mRNA was induced by T_3, and the largest increase was seen at 24 h (Fig. 8B). There were no effects of CORT on GR mRNA in XLT-15 cells. We could not detect D2 or D3 mRNA in this cell line; TRα and MR were not investigated.

4. Discussion

Earlier studies showed that CS can synergize with TH to promote tadpole tail resorption, and biochemical analyses supported that CS can enhance TH action by upregulating TRs and 5′ deiodinase (reviewed by Kikuyama et al., 1993). Many of these studies used doses of CS that would be expected to produce supraphysiological tissue hormone content (e.g., Gray and Janssens, 1990), used the superactive glucocorticoid analog dexamethasone (DEX), or were not replicated, and therefore precluded quantitative or statistical analysis (e.g., Iwamuro and Tata, 1995). Here we provide a molecular basis for synergy among CS and TH in tadpole metamorphosis through analysis of gene expression using several experimental paradigms and doses of CORT that we previously showed produce tissue hormone content within the physiological range. A major effect of CS is at the level of the TRβ gene, whose mRNA was increased in a synergistic way by combined treatment with T_3 and CORT. This effect on TRβ was seen in tail explants, in tadpole tail and brain *in vivo*, and in two *X. laevis* tissue culture cell lines, XTC-2 and XLT-15. Furthermore, the kinetics in XTC-2 cells was rapid, with significant potentiation of mRNA levels by 4 h of hormone treatment. A second basis for the synergy is at the level of D2 gene expression, where CORT induced D2 mRNA, or acted in synergy with T_3. Thus, CS may enhance tissue sensitivity to TH by upregulating expression of TRβ, increasing cellular responsive-

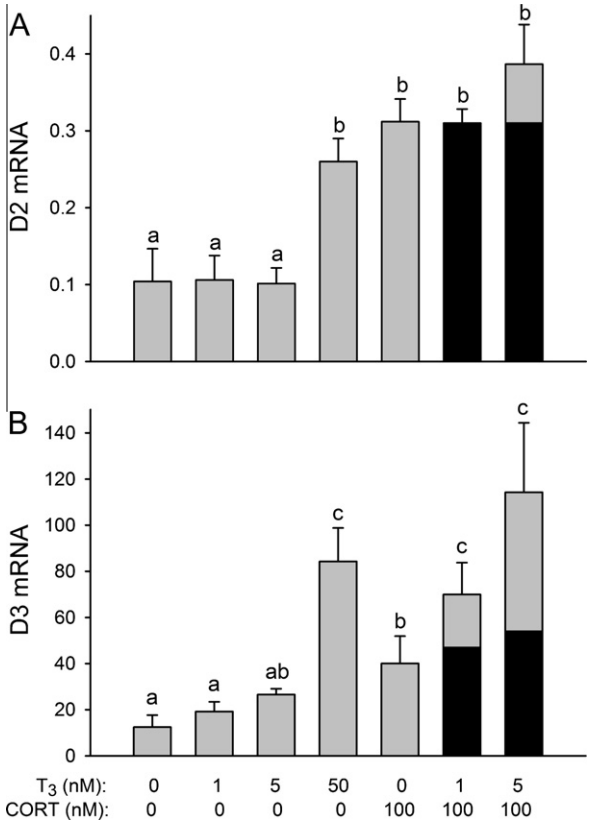

Fig. 5. Effects of corticosterone (CORT) and 3,5,3′-triiodothyronine (T_3) alone or in combination on expression of deiodinase type 2 (D2; A) and type 3 (D3; B) mRNAs in tail from tadpoles treated with hormones *in vivo*. Nieuwkoop–Faber stage 52 *S. tropicalis* tadpoles were treated with T_3 and CORT at the doses indicated for 24 h before sacrifice as described in Section 2. Gene expression was analyzed by RTqPCR using Taqman assays. The data are given as ratios of gene expression to rpL8; the normalized gene expression data were $\log_{10}$-transformed before statistical analysis using ANOVA. The letters indicate significant differences between the group means based on Fisher's LSD ($P < 0.05$; $n = 5$/treatment). The black bars represent the estimated additive responses to T_3 or CORT alone at the doses indicated.

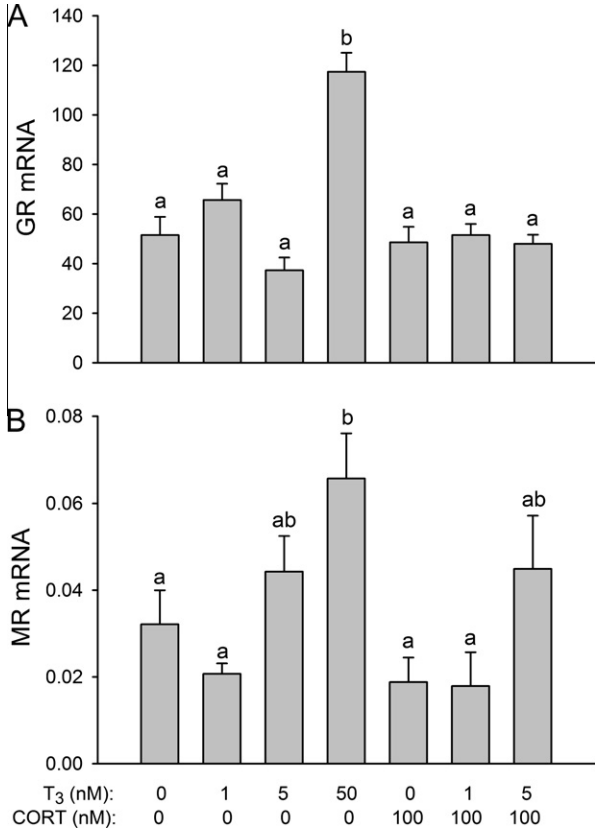

Fig. 6. Effects of corticosterone (CORT) and 3,5,3′-triiodothyronine (T_3) alone or in combination on expression of glucocorticoid receptor (GR; A) and mineralocorticoid receptor (MR; B) mRNAs in tail from tadpoles treated with hormones *in vivo*. Nieuwkoop–Faber stage 52 *S. tropicalis* tadpoles were treated with T_3 and CORT at the doses indicated for 24 h before sacrifice as described in Section 2. Gene expression was analyzed by RTqPCR using Taqman assays. The data are given as ratios of gene expression to rpL8; the normalized gene expression data were $\log_{10}$-transformed before statistical analysis using ANOVA. The letters indicate significant differences between the group means based on Fisher's LSD ($P < 0.05$; $n = 5$/treatment). The black bars represent the estimated additive responses to T_3 or CORT alone at the doses indicated.

ness to the hormone, and by upregulating D2, thereby promoting generation of the more biologically active form of the hormone T_3.

The potentiating actions of CS on TH-induced tadpole metamorphosis have been known since the mid-1950s, and were first described by Frieden and Naile (1955) who showed that hydrocortisone accelerated both T_4 and T_3-induced metamorphosis in three anuran species. Similar findings were obtained by others with tadpoles of different anuran species including *X. laevis* (Gray and Janssens, 1990; reviewed by Denver, 2009a; Kikuyama et al., 1993). A physiological role for CS in tadpole metamorphosis is supported by the findings of Kikuyama et al. (1982) who showed that treatment of tadpoles with the CS synthesis inhibitor amphenone B retarded T_4-induced tail resorption, and that this could be reversed by treatment with CS. Several investigators reported synergistic actions between CS and TH on regression of tadpole tail explants (Kikuyama et al., 1993; Kikuyama et al., 1983). Gray and Janssens (1990) found synergy between T_3 (50 nM) and CORT (3400 nM) in *X. laevis* tadpole tail explants; lower doses of CORT were not tested. We found that doses of CORT (100 and 500 nM) that were previously shown to produce tissue content within the physiological range for amphibians caused significant synergistic actions with T_3 on tadpole tail regression *in vitro*, thus supporting a physiological role for CS in tadpole tail regression. Furthermore, these actions were seen with a dose of T_3 (10 nM) that by itself had no effect on tail regression, which provides convincing evidence for hormone synergy.

Work from Kikuyama's laboratory showed that CS increase nuclear binding capacity for T_3 in tadpole tail (Niki et al., 1981; Suzuki and Kikuyama, 1983). The frog TRβ gene is auto-induced during metamorphosis (reviewed by Tata, 2006). The synergy that we saw among T_3 and CORT on regression of tadpole tail explants was paralleled by a simultaneous, synergistic upregulation of TRβ mRNA. This synergy was also seen in the tail and brain of tadpoles treated with hormones *in vivo*, and in both XTC-2 and XLT-15 cells. Iwamuro and Tata (1995) reported that the potent glucocorticoid DEX (they used a dose roughly equivalent to 1000 nM CORT) enhanced T_3-dependent TRβ expression in tadpole tail explants and XTC-2 cells. There are other examples where CS potentiates TH actions on gene expression. For example, in rodents, TH and CS act synergistically to induce growth hormone gene expression in pituitary and pituitary cell lines (Dobner et al., 1981; Martial et al., 1977; Nogami et al., 1995; Samuels et al., 1977; Shapiro et al., 1978). Glucocorticoids also act synergistically to increase somatotrope abundance in the rat (Hemming et al., 1988) and chick embryonic pituitary gland, and in the chick this may be attributed to an increase in 5′ deiodinase activity (Liu et al., 2003). Similar synergy between TH and CS was observed on several hepatic genes in mammals (Menjo et al., 1993; Molero et al., 1993; Yamaguchi et al., 1999).

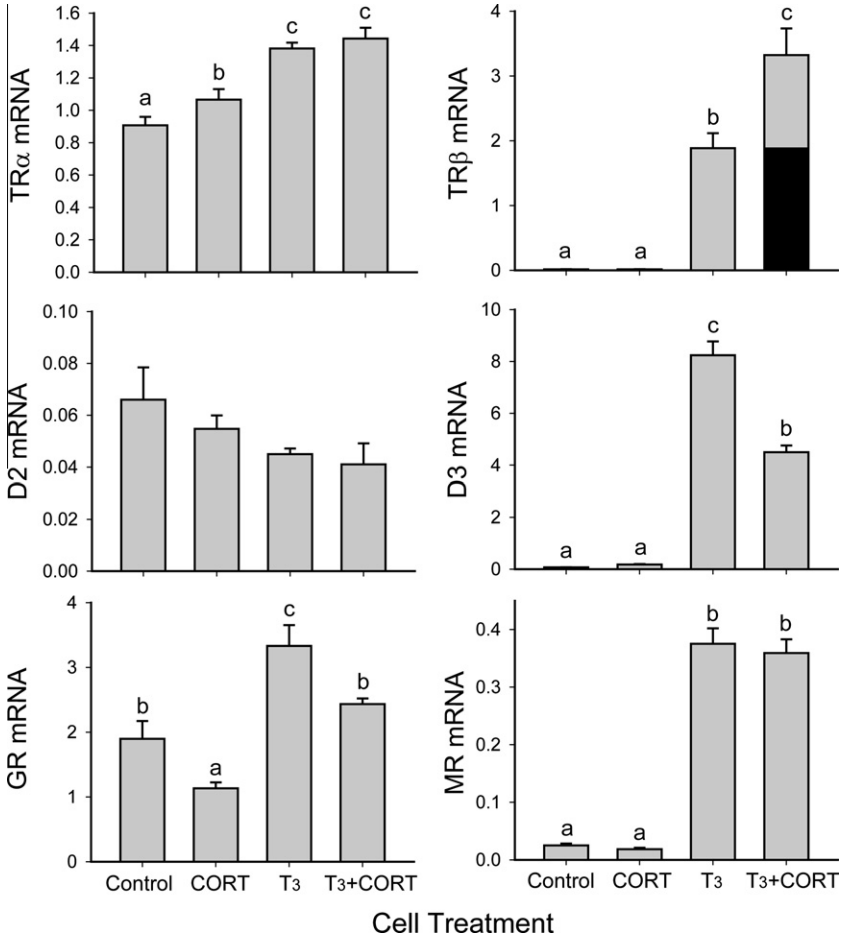

Fig. 7. Effects of corticosterone (CORT) and 3,5,3′-triiodothyronine (T_3) alone or in combination on expression of thyroid hormone receptor alpha (TRα), TRβ, deiodinase type 2 (D2), D3, glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) mRNAs in the fibroblast-derived *X. laevis* cell line XTC-2. Cells were treated with or without hormones for 4 h before harvest and RNA isolation as described in Section 2. Gene expression was analyzed by RTqPCR using Taqman assays. The data are given as ratios of gene expression to rpL8; the normalized gene expression data were $\log_{10}$-transformed before statistical analysis using ANOVA. The letters indicate significant differences between the group means based on Fisher's LSD ($P < 0.05$; $n = 5$/treatment). The black bar on the TRβ graph represents the estimated additive responses to T_3 or CORT alone at the doses indicated.

While the autoinduction of TRβ is explained by the presence of TH response elements (TREs) located in the proximal promoter region of the gene (Ranjan et al., 1994; Machuca et al., 1995), we do not yet understand the molecular basis for synergy between T_3 and CORT on TRβ. One hypothesis is that CS responsive elements are present in the TRβ promoter region perhaps adjacent to or overlapping with the TREs. However, we found no evidence that CS could modulate TRβ promoter activity in transient transfection assays using XTC-2 or XLT-15 cells (E.D. Hoopfer, C. Kholdani and R.J. Denver, unpublished data; see also Iwamuro and Tata, 1995). This suggests that (1) CS responsive elements are located elsewhere in the TRβ locus, or (2) CS induce the expression of a transcription factor that enhances TRβ autoinduction. It is also possible that CS act posttranscriptionally to stabilize TRβ mRNA. A direct transcriptional mechanism of action may occur in mammals where CS increased nuclear T_3 binding capacity and TRβ1 transcription in rat liver (Montesinos et al., 2006). Montesinos et al. (2006) also provided some evidence that CS action on TRβ1 transcription was due to direct binding and transactivation of the TRβ promoter by GR. Synergy between TR and GR was reported in rat pituitary tumor cells and required the GR DNA-binding domain and was mediated by the GR transactivation domains (Leers et al., 1994).

The kinetics of synergistic TRβ upregulation by T_3 plus CORT was rapid in XTC-2 cells suggesting direct actions of CS on TRβ transcription in tadpoles. However, we cannot rule out that the actions of CS were due to upregulation of a transcription factor that regulates the TRβ gene rather than direct modulation of TRβ by GR or MR. A candidate for this transcription factor is the immediate early gene Krüppel-like factor 9 (KLF9; also known as basic transcription element binding protein 1; BTEB1). Krüppel-like factor 9 is the most rapidly responding TH-induced gene thus far identified in tadpole tissues, and its expression parallels the increase in plasma TH that occurs during tadpole metamorphosis (Denver et al., 1997; Furlow and Kanamori, 2002; Hoopfer et al., 2002; Shi, 2000; Wang and Brown, 1993). The TH responsiveness of the frog gene can be attributed to a TRE located ~6.5 kb upstream of the transcription start site of the *X. laevis* KLF9 gene (Furlow and Kanamori, 2002). Our previous work showed that KLF9 functions as an accessory transcription factor for TRβ autoinduction (Bagamasbad et al., 2008). We also found that KLF9 is strongly induced by CORT in juvenile frog (Bonett et al., 2009) and tadpole brain (F. Hu and R.J. Denver, unpublished data). Indeed, KLF9 is synergistically upregulated by TR and GR via a highly conserved 'synergy module' located in the 5′ flanking region of frog and mouse genes (P. Bagamasbad and R.J. Denver, unpublished data). Therefore, rapid CS upregulation of KLF9 could be responsible for the synergistic activation of TRβ by T_3 and CORT.

Another possibility for a CS-regulated transcription factor is retinoid X receptor (RXR) which heterodimerizes with TR on positive TREs. Iwamuro and Tata (1995) reported that while T_3 alone down-

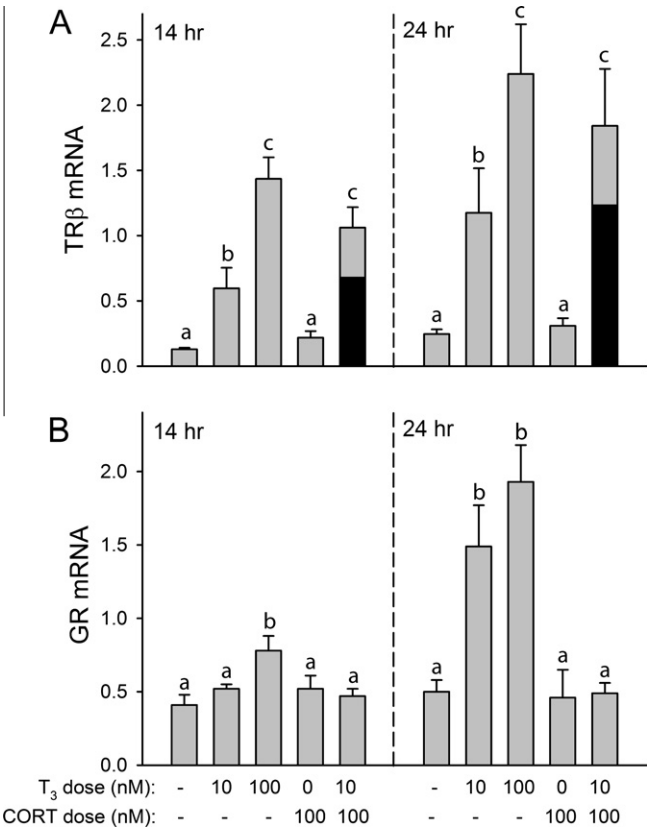

Fig. 8. Effects of corticosterone (CORT) and 3,5,3′-triiodothyronine (T_3) alone or in combination on expression of thyroid hormone receptor beta (TRβ) and glucocorticoid receptor (GR) mRNAs in the *X. laevis* tadpole tail myoblast-derived cell line XLT-15. Cells were treated with or without hormones for 14 or 24 h before harvest and RNA isolation as described in Section 2. Gene expression was analyzed by semi-quantitative RT-PCR. The data are given as ratios of gene expression to rpL8; the normalized gene expression data were $\log_{10}$-transformed before statistical analysis using ANOVA. The letters indicate significant differences between the group means based on Fisher's LSD ($P < 0.05$; $n = 6$/treatment). The black bars represent the estimated additive responses to T_3 or CORT alone at the doses indicated.

regulated RXRγ mRNA in *X. laevis* tadpole tail explants, DEX alone increased expression (but T_3 suppressed the DEX effect when the two hormones were combined). Treatment of rat hepatocytes with DEX potentiated T_3 effects on type 1 deiodinase, Spot 14 and malic enzyme mRNAs (Menjo et al., 1993; Yamaguchi et al., 1999). DEX alone increased RXRα mRNA, and these authors proposed that the enhancement of T_3 action was due to the upregulation of RXRα.

Although TRα exhibited a similar pattern of response to TRβ in tadpole tail explants, these effects were subtle and did not reach statistical significance. However, we found statistically significant responses to hormone treatment of TRα mRNA in tail of tadpoles treated *in vivo*, and in XTC-2 cells. In these experiments TRα mRNA was induced by CORT or by T_3, and was synergistically induced by the higher dose of T_3 (5 nM) in tadpole tail *in vivo*, but not in XTC-2 cells at 4 h. Taken together, our results suggest that the more significant synergistic regulation by T_3 and CORT occurs on TRβ as compared with TRα.

The intracellular concentration of T_3 is modulated by the activities of D2 and D3 (St Germain et al., 2009). Our findings support that a secondary mechanism for CORT to enhance tissue responsiveness to TH is through upregulation of D2, either alone or in synergy with T_3. We consider this to be secondary because the hormonal response of D2 may be delayed as compared with the immediate and strong upregulation of TRβ. We found that CORT alone could induce D2 mRNA in tadpole tail *in vivo* but there was no additive or synergistic effects of combined treatment with T_3 plus CORT at the single time point measured (24 h; see Fig. 4A). In tadpole tail explants the expression of D2 mRNA was induced by combined treatment with T_3 (10 nM) plus CORT (500 and 3400 nM) at both time points investigated (48 and 120 h). Although we detected D2 mRNA in XTC-2 cells it was not induced by T_3 by 4 h, which may reflect that D2 is a delayed response gene (discussed below). Thus, our data suggest that T_3 and CORT can promote expression of D2 in tadpole tail. The action of T_3 is consistent with earlier findings that showed that TH positively regulates 5′ deiodinase activity, and D2 mRNA in tadpoles (Brown, 2005; Buscaglia et al., 1985; Hogan et al., 2007). Our finding that CORT induced D2 mRNA in tadpole tail *in vivo* agrees with the findings of Galton (1990) who reported that treatment of bullfrog tadpoles with 1 μM CORT in their aquarium water increased 5′ deiodinase activity in skin.

The 5 and 5′ deiodinase activities, and D2 and D3 mRNAs show tissue-specific and developmental stage-specific expression patterns in tadpoles that reflect the asynchronous tissue morphogenesis that occurs during metamorphosis, and the roles that the deiodinases play in modulating TH action (Brown, 2005; St Germain et al., 2009). Both enzymes may be co-expressed in cells, the relative expression levels providing a push–pull mechanism to regulate intracellular T_3 concentrations (Becker et al., 1997), or they may be expressed at different times of development. In the tadpole tail D3 mRNA is found in several cell types including tail fibroblasts, but not muscle cells (Berry et al., 1998), and D2 is expressed primarily in fibroblasts (Cai and Brown, 2004). D3 mRNA (and 5 deiodinase activity) is expressed in the tail throughout tadpole life, increases during late prometamorphosis (NF stage 59–61) but then declines sharply at metamorphic climax (Brown et al., 1996; St Germain et al., 1994). The tadpole tail is an essential locomotory organ, and this pattern of D3 expression is hypothesized to protect the tail from the actions of T_3 before the animal reaches an appropriate stage of development to initiate tail regression (Brown, 2005). By contrast, D2 expression is low or non-detectable before metamorphic climax (NF stage 62) when its expression increases strongly and continues through NF stage 64 (Cai and Brown, 2004). Our failure to detect D2 or D3 mRNA in the tail myoblast-derived XLT-15 cells is consistent with *in situ* hybridization studies that showed that these genes are not expressed in tail muscle cells. The expression of D2 and D3 in non-muscle tail cells suggests that these cells either destroy bioactive TH to protect tail muscle cells from apoptosis (when D3 is expressed), or generate a high local concentration of T_3 to act on tail muscle cells to cause tail regression during metamorphic climax (when D2 is expressed). This situation is analogous to that in mammalian brain, where astrocytes, but not neurons, express deiodinases and therefore control exposure of neurons to bioactive TH (Trentin, 2006).

The increase in D3 expression is coincident with a rise in circulating T_3 during prometamorphosis (Leloup and Buscaglia, 1977). The 5 deiodinase activity and D3 mRNA are induced by TH in tail (Brown et al., 1996; St Germain et al., 1994; Wang and Brown, 1993), brain (Denver et al., 1997; Hogan et al., 2007) and in XTC-2 cells (St Germain et al., 1994). We also observed this regulation of D3 in tadpole tail and brain, and the early response kinetics in XTC-2 cells supports that D3 is a direct TH target gene (Das et al., 2006; St Germain et al., 1994). We observed a small increase in D3 mRNA in the tails from tadpoles treated with CORT for 24 h, and also synergy with T_3. However, in XTC-2 cells CORT alone had no effect, but CORT reduced the response to T_3 in D3 mRNA. Galton (1990) found that treatment of bullfrog tadpoles with CORT for 48 h reduced 5 deiodinase activity in tadpole liver and gut, and Lorenz et al. (2009) reported that treatment of *X. laevis* tadpoles with CORT or dexamethasone for 21 days decreased D3 mRNA. We observed a trend towards decreasing D3 mRNA with CORT in

tadpole tail explants at both time points. In the chicken, glucocorticoids have been shown to downregulate 5 deiodinase activity and D3 mRNA (reviewed by De Groef et al., 2008). Taken together, the findings suggest that the actions of CS on D3 expression are tissue-specific and time-dependent. CORT may synergize with TH to upregulate D3 mRNA in tail as an early response (by contrast to its inhibitory effect on T_3-induced upregulation of D3 mRNA in XTC-2 cells). However, over a longer period, D3 may be repressed by CS in most tissues. For reasons that are unknown, the expression of the D3 gene is decreased at metamorphic climax when TH concentrations are highest. Expression of other direct TR target genes (e.g., KLF9) continues to increase in the tadpole tail through metamorphic climax (Brown et al., 1996). This is a time when plasma CS are also rising in tadpoles (reviewed by Denver, 2009a), which suggests the hypothesis that the late decrease in D3 is related to the elevated CS.

Unlike D3 which shows early response kinetics, D2 appears to be a delayed response gene. Brown (2005) commented that the upregulation of D2 mRNA caused by T_3 required several days of hormone treatment, and Das et al. (2006) did not find D2 to be a direct T_3 target in microarray studies. Our results in tadpole tail explants are consistent with these findings in that we saw only a small effect of T_3 on D2 mRNA at 48 h but clear upregulation by 120 h. However, when we treated tadpoles with T_3 (50 nM) we saw upregulation of D2 mRNA by 24 h in the tail and in the brain; we did not examine earlier timepoints. More study is needed to understand the mechanism by which D2 gene expression is regulated by TH in *X. laevis* and *S. tropicalis*.

Another important point of regulation in this system is the induction of GR and MR expression by T_3, which would be expected to increase tissue sensitivity to further actions of CS. We found that treatment of tadpoles with T_3 induced GR and MR mRNA in tail. We obtained similar results in XTC-2 cells for both genes, and in tail explants and XLT-15 cells for GR. The positive effect of T_3 on GR in tail in *S. tropicalis* is consistent with our previous findings in *X. laevis* (Krain and Denver, 2004). CORT treatment did not affect GR or MR mRNA in tadpole tail *in vivo* or in tail explants. However, GR was negatively regulated by CORT in XTC-2 cells; GR is negatively regulated by T_3 and CORT in tadpole brain (Krain and Denver, 2004; Yao et al., 2008).

By contrast to the synergistic effect of combined treatment with T_3 plus CORT, treatment of tadpole tail explants with CORT alone tended to increase final tail dry weight which was statistically significant for the highest CORT dose. We do not know whether this effect is of physiological significance. However, it is worth noting that CORT treatment *in vivo* increased tail size of *Rana pipiens* (Glennemeier and Denver, 2002) and *Rana sylvatica* tadpoles (J. Middlemis-Maher, E.E. Werner, and R.J. Denver, unpublished data). The mechanism for this effect is unknown, but it may have functional importance with regard to predation, where chronic predator presence elevates CORT in premetamorphic tadpoles (J. Middlemis-Maher, E.E. Werner, and R.J. Denver, unpublished data). Tadpoles of many amphibian species exposed to predation develop larger tails (Relyea, 2007), and this increased tail size could be blocked with the CS synthesis inhibitor metyrapone (J. Middlemis-Maher, E.E. Werner, and R.J. Denver, unpublished data).

In summary, TH and CS act synergistically to cause regression of the tadpole tail. This synergy may be explained by the cooperative upregulation of TRβ and D2 expression which should increase tissue sensitivity to T_3, and enhance conversion of T_4 to T_3, the more biologically active form of the hormone. Concurrently, CS may cause early upregulation of D3, an adaptive response to protect the tail from the apoptotic actions of T_3, but with prolonged exposure cause downregulation of D3 to promote tail regression. Thus, the early response to hormones is to upregulate TRβ and D3, the delayed response is to upregulate D2 and to downregulate D3. The TRβ gene expression response is sustained and amplified due to autoinduction (which is favored by the synergistic upregulation of the immediate early gene KLF9), synergy with CS, and enhanced generation of T_3 in target tissues. The CS response is also enhanced by the actions of T_3 on the expression of GR and MR.

The ecological/organismal significance of this hormonal gene regulation during development is that activation of the stress axis in a tadpole by environmental stressors would (1) lead to increased production of TH and CS (through increased CRF secretion), and (2) enhance tissue sensitivity of target tissues to the TH signal through the actions of CS. Together, these two hormonal systems may cooperate to accelerate metamorphosis under conditions of ecological stress (e.g., pond drying, competition for resources, predation, habitat degradation, etc.)

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygcen.2010.03.014.

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