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Research paper

In-vivo regulation of Krüppel-like factor 9 by corticosteroids and their receptors across tissues in tadpoles of *Xenopus tropicalis*

Leena H. Shewade, Katelin A. Schneider, Audrey C. Brown, Daniel R. Buchholz *

Department of Biological Sciences, University of Cincinnati, 312 Clifton Court, Cincinnati, OH 45221, USA

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ABSTRACT

Corticosteroids are critical for normal development and for mediating effects of stress during development in all vertebrates. Even though gene knockout studies in mouse and zebrafish have identified a number of developmental roles of corticosteroids and their receptors, the numerous pleiotropic actions of these hormones affecting various aspects of development are understudied. For the most part, neither the endogenous hormone(s) nor their receptor(s) regulating developmental processes during natural development have been determined. Here, we address this issue by elucidating the endogenous regulation of the transcription factor *Krüppel-like factor 9* (*klf9*) across tissues during development by corticosteroid hormones (aldosterone and corticosterone) and their nuclear receptors (type-I and type-II receptors). First, we measured the developmental expression profiles of *klf9* and type-I and type-II corticosteroid receptors in key target tissues, brain, lungs, and tail, during larval and metamorphic stages in *Xenopus tropicalis*. We also studied the corticosteroid regulation of *klf9* in these tissues *in-vivo* using exogenous hormone treatments and receptor antagonists. *Klf9* and the corticosteroid receptors were expressed in each tissue and significantly increased in expression reaching a peak at metamorphic climax, except for the type-II receptor in brain and tail whose expression did not change significantly across stages. Both corticosteroid hormones induced *klf9* in each tissue, although aldosterone required a five times higher dose than corticosterone to cause a significant induction. The upregulation of *klf9* by both corticosteroids was completely blocked by the use of the type-II receptor antagonist RU486 and not the type-I receptor antagonist spironolactone. These results are consistent with previous *in-vitro* studies and indicate for the first time *in-vivo* that corticosteroid regulation of *klf9* occurs exclusively via corticosterone and type-II receptor interaction across tissues.

1. Introduction

Corticosteroids are primary vertebrate stress hormones that play vital roles during development. They are responsible for organ maturation in preparation for life history transitions, such as birth and metamorphosis, and for mediating the effect of the environment through altered timing of organ maturation (Fowden and Forhead, 2015; Fowden et al., 1998; Khulan and Drake, 2012; Wada, 2008). The two corticosteroids, aldosterone and corticosterone (or cortisol in humans), act via two nuclear receptor proteins, type-I receptor (also known as the mineralocorticoid receptor, MR) and type-II receptor (also known as the glucocorticoid receptor, GR), to regulate gene expression underlying developmental responses to corticosteroids. While the type-I receptor binds with a very high affinity to both ALDO and CORT, the type-II receptor shows a comparatively lower affinity to CORT and does not bind ALDO at physiological concentrations (Funder, 1997; Roubos et al., 2009).

Gene knockout studies have begun to sort out which corticosteroid hormone and which receptor are responsible for specific corticosteroid-dependent developmental events. Mice lacking the type-II receptor die at birth due to lung atelectasis, but these mice also show severely impaired adrenal glands and decreased gluconeogenic activity (Cole et al., 1995). Brain-specific type-II receptor mutants only show behavioral abnormalities characteristic of anxiety and learning phenotypes (Tronche et al., 1999). Type-I receptor knockout mice show increased hypothalamic hormone activity suggestive of its role in corticosteroid feedback regulation as well as reduced neurogenesis (Berger et al., 1998, 2006; Gass et al., 2000, 2001). A zebrafish type-II receptor knockout model shows only a moderate stress behavior phenotype suggesting a possible compensatory mechanism (Griffiths et al., 2012). The complexity and pleiotropy of the developmental actions by

* Corresponding author.
E-mail address: buchhodr@ucmail.uc.edu (D.R. Buchholz).

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corticosteroids (Fowden and Forhead, 2015) leaves the specific hormone or receptor responsible for many stress and corticosteroid actions difficult to determine. Type-I receptor knockdowns in mice cause increased type-II receptor activity compounding the difficulty in identifying specific developmental roles for the two corticosteroid hormones and their receptors (Berger et al., 2006).

The dramatic effects of hormones on amphibian metamorphosis provide a valuable model to examine the role of stress hormones and their receptors in development (Buchholz, 2015). However, few if any corticosteroid-dependent events during metamorphosis have been attributable to a specific hormone/ligand interaction. The amphibian stress hormone, corticosterone (CORT), is believed to be the hormonal mediator of the environmental effects of stress because an increase in CORT in response to stress has been measured in a number of tadpole species (Bonett et al., 2010; Hu et al., 2008; Krain and Denver, 2004; Middlemis Maher et al., 2013; Roubos et al., 2009; Yao et al., 2008). Also, *in-vivo* and *in-vitro* studies have shown that exogenous CORT can synergize with exogenous thyroid hormone to accelerate metamorphic events, while type-II receptor antagonists and CORT synthesis blockers reduce the effects of stress on metamorphosis (Bagamasbad et al., 2012; Bonett et al., 2009; Kulkarni and Buchholz, 2012). However, exogenous treatment with ALDO similarly affects thyroid hormone-dependent metamorphic development (Jolivet Jaudet and Leloup Hatey, 1984; Kikuyama et al., 1983, 1986; Ui et al., 1983; Niki et al., 1981). Also, plasma levels of both CORT and ALDO rise to a peak at metamorphic climax (Jolivet Jaudet and Leloup Hatey, 1984). Importantly, the pituitary hormone ACTH, which is responsible for CORT production, also induces ALDO production when injected into tadpoles (Krug et al., 1983; Macchi and Phillips, 1966), as in mammals (Funder, 2016). So far, no study has conducted measurements of ALDO after stress treatments in tadpoles. Thus, ALDO and/or CORT could be responsible for corticosteroid-dependent developmental effects.

In an effort to identify specific roles for corticosteroids in mediating developmental effects during metamorphosis, we here examine the regulation via corticosteroids and their receptors of a known CORT-response gene, *kruppel-like factor 9* (*klf9*) in tadpoles undergoing metamorphosis. No other CORT-response gene is well-described in tadpoles. *Klf9* is a transcription factor induced by both thyroid hormone and CORT individually and synergistically (Bagamasbad et al., 2012; Bonett et al., 2009; Hoopfer et al., 2002). *Klf9* is known for its developmental role in response to stress in the brain of *X. laevis* tadpoles as well as mice, where it can alter neuronal structure and differentiation (Bagamasbad et al., 2012; Bonett et al., 2009). The developmental expression of *klf9* has been shown for several tadpole tissues, including brain, tail, and intestine, and treatment with CORT induces *klf9* expression in all tadpole tissues previously examined (Hoopfer et al., 2002). The induction of *klf9 in-vivo* by ALDO has not been tested.

Both nuclear receptors for corticosteroids are expressed in all tadpole tissues tested, namely tail and pituitary for type-I receptor and tail, brain, and intestine for type-II receptor (Bonett et al., 2010; Krain and Denver, 2004; Roubos et al., 2009; Thurmone et al., 1986). An enhancer element upstream of *kfl9* has been identified in frogs and mammals as a CORT and thyroid hormone synergy module containing hormone response elements for thyroid hormone and corticosteroids (Bagamasbad et al., 2012). Because hormone response elements for type-I and type-II receptors in corticosteroid-regulated genes have the same sequence motif (Funder, 1997; Pearce and Yamamoto, 1993), it is not clear which corticosteroid/receptor interaction(s) regulate *klf9* expression *in-vivo*.

Studies using type-I- and type-II-specific agonists and antagonists have provided insight into the corticosteroid regulation of *klf9* (Bonett et al., 2009). In juvenile *Xenopus* (tadpoles were not tested), stress-induced induction of *klf9* in the brain was completely or almost completely blocked using RU486 (a type-II receptor specific antagonist) pointing to the use of the type-II receptor, at least in post-metamorphic stages (Bonett et al., 2009). Using cell lines derived from *Xenopus* and mouse, CORT-induced *klf9* expression was almost completely suppressed by RU486 but mostly not by spironolactone (a type-I receptor specific antagonist) (Bagamasbad et al., 2012; Bonett et al., 2009). Further, dexamethasone (a type-II receptor-specific agonist) can induce *klf9* and be blocked by RU486 in cell lines and mouse primary cerebrocortical

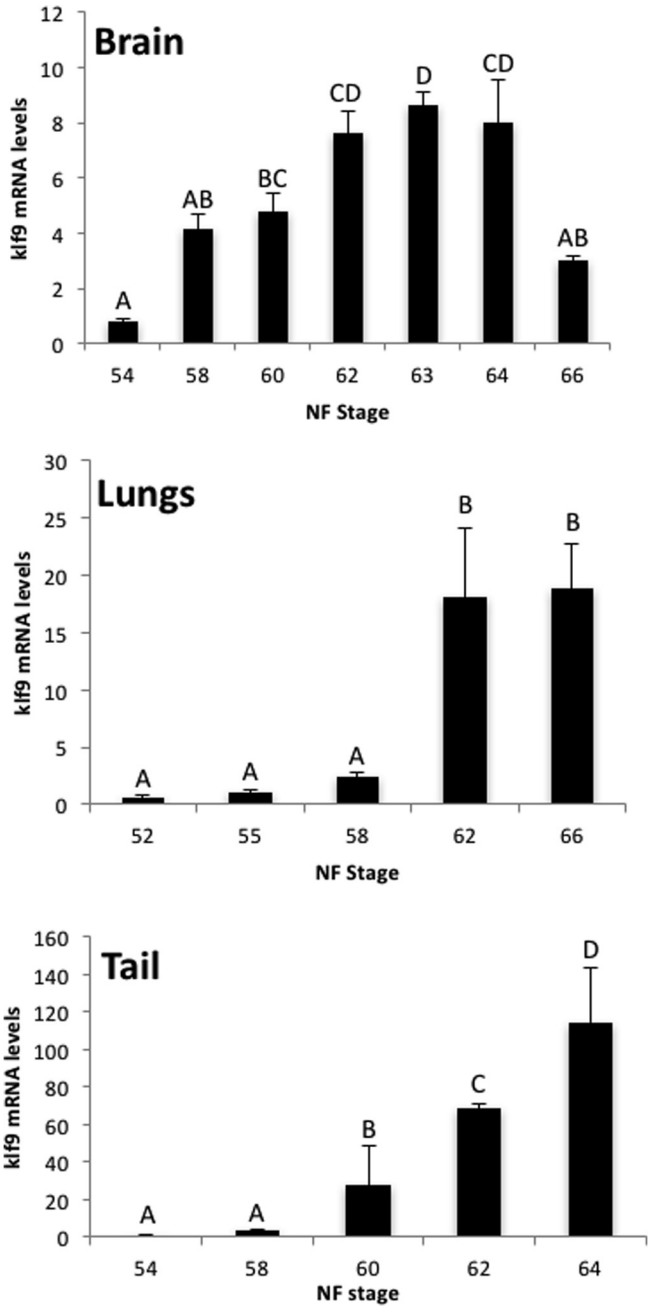

Fig. 1. Developmental profile of *klf9* expression in the brain, lungs, and tail during *Xenopus* metamorphosis. The expression of *klf9* increased significantly during metamorphosis in each tissue. Total RNA was collected from tadpole tissues at the indicated developmental stages to measure *klf9* mRNA expression by quantitative PCR. Bars show the mean mRNA levels relative to the reference gene *rpL8*. Error bars represent SEM. The letters above the error bars indicate significance groups among stages based on Tukey's honest significant difference test ($p < 0.05$, $n = 5$ for brain and tails and $n = 3$ for lungs per stage).

cells (Bagamasbad et al., 2012; Gil-Ibáñez et al., 2014). In a mouse cell line overexpressing type-I receptor, ALDO can induce *klf9*, including at concentrations likely too low to activate type-II receptors though not always significantly (Bagamasbad et al., 2012). Also, in a different mouse cell line overexpressing type-I receptor and transfected with a *klf9* enhancer reporter construct, ALDO-induced reporter expression was partially blocked by RU26752 (a type-I receptor-specific antagonist) (Bagamasbad et al., 2012). These data, from juvenile frogs and *in-vitro* brain-derived cell culture studies, suggest that the CORT-type II receptor interaction is the main if not only contributor to the induction of *klf9* with equivocal evidence for a possible role of ALDO and type-I receptor, but in any case the *in-vivo* situation during development has not been determined.

Here, to gain insight into the role of corticosteroids during metamorphosis, we examined the developmental expression profiles of type-I and type-II receptors as well as *klf9* in key CORT target tissues (brain, lungs, and tail) in tadpoles across developmental stages through metamorphosis. We then tested the effect of corticosteroid hormone treatments and receptor antagonists on *klf9* expression to examine the possible *in-vivo* action of corticosteroids and their receptors on the regulation of *klf9* expression among tissues.

2. Materials and methods

2.1. Animal husbandry

Lab-reared male and female adult *Xenopus tropicalis* were mated by priming with 20 U of human chorionic gonadotropin (Sigma) in the evening and boosting with 200 U the next morning. Resulting tadpoles were reared at 26 C and fed Sera Micron twice daily with daily water changes. The use of animals in experiments was in accordance with the guidelines of University of Cincinnati

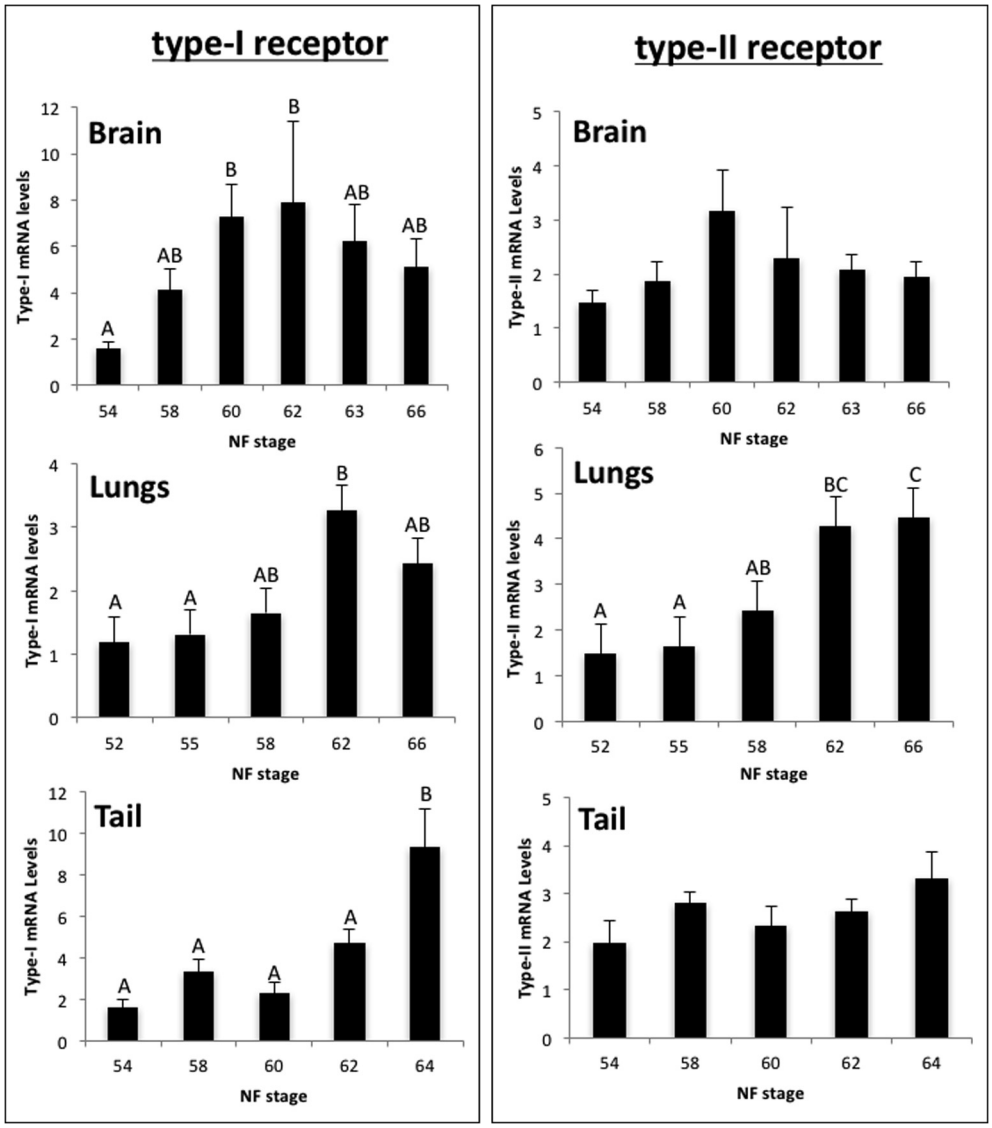

Fig. 2. Developmental profiles of type-I and type-II corticosteroid receptor expression in the brain, lungs, and tail during *Xenopus* metamorphosis. Expression of both receptors was detectable in each tissue at each stage and increased significantly during metamorphic climax in most cases. Total RNA was extracted from tadpole tissues at the indicated developmental stages to measure receptor mRNA expression by quantitative PCR. Bars show the mean mRNA levels relative to the reference gene *rpL8*. Error bars indicate SEM. The letters above the error bars indicate significance groups among stages based on Tukey's honest significant difference test ($p < 0.05$, $n = 5$ for brain and tail and $n = 3$ for lungs per stage). No letters above bars indicates a lack of significant difference among stages within the tissue.

Institutional Animal Care and Use Committee (IACUC protocol # 06-10-03-01).

2.2. Hormone treatments and gene expression analysis

For the developmental expression profiles of *klf9* and type-I and type-II receptors, brain, lungs, and tail were harvested from tricaine-anaesthetized tadpoles at developmental stages throughout the larval period and metamorphosis, staged according to Nieuwkoop and Faber (NF) with n = 5–10 per tissue per stage (Nieuwkoop and Faber, 1956). For the dose response experiment, tadpoles at the end of premetamorphosis (NF54), were treated for 24 h with 100 nM or 500 nM of CORT or ALDO (Acros Organics) or EtOH vehicle control by addition to aquarium water before harvesting tails (n = 10 per experimental condition). For the receptor antagonist experiments with CORT treatments, brain, lungs, and tail were harvested from premetamorphic tadpoles (NF 54) treated for 24 h with i) EtOH control, ii) 100 nM CORT, iii) 100 nM CORT + 150 nM RU486 (Apexbio Technologies LLC), iv) 100 nM CORT + 150 nM spironolactone (Alfa Aesar), v) 100 nM CORT + 150 nM RU486 + 150 nM spironolactone, vi) 150 nM RU486, and vii) 150 nM spironolactone (n = 10 per experimental group). The antagonist doses were set to 50% more than the hormone to block the receptor binding (Rollins-Smith et al., 1997). A parallel receptor antagonist experiment was repeated for ALDO, except 500 nM ALDO was used, only tails were harvested, and the antagonist-only treatments were not repeated. Tissues were snap frozen immediately following harvest on dry ice and stored at −80 C. For brain and lungs, two and three individuals, respectively, were pooled together to obtain enough RNA. Total RNA extraction was performed using TriReagent (Molecular Research Centre Inc.) following manufacturer's instructions. One µg of RNA was used per sample to synthesize cDNA (Biotool Inc) using manufacturer's protocol. One µl of cDNA was used in 20 µl reactions for quantitative PCR with FAM labeled primer-probe sets and Taqman Universal master mix (Life technologies) on 7300 Real time PCR system, using PCR conditions and primer/probe sequences as previously described (Choi et al., 2014). The relative quantification method was used to measure the expression levels of *klf9* normalized to the reference gene *rpl8* (Livak and Schmittgen, 2001).

2.3. Statistical analysis

Data analysis was performed using JMP Pro 12 statistical analysis software. Analysis of Variance (ANOVA) was performed to identify significant differences in *klf9* expression among developmental stages and among hormone and antagonist treatments. A *p*-value less than 0.05 was considered to be statistically significant. Pair-wise comparisons were done using the Tukey-Kramer *post-hoc* test. All error bars indicate standard error of the mean. All hormone treatment experiments show results from a single experiment, which were independently repeated 2–4 times using different clutches of offspring with similar results.

3. Results

3.1. Developmental expression profile of klf9

To confirm the expected expression of *klf9* among tadpole tissues, we measured *klf9* mRNA levels in brain, lungs, and tail of *X. tropicalis* tadpoles throughout metamorphosis from premetamorphosis (NF54) through metamorphic climax (NF62) to tail resorption (NF66) including intervening stages (Fig. 1). The levels of *klf9* peaked at metamorphic stages in the brain (Fig. 1A), mimicking the profiles of thyroid hormone and CORT content in the blood, which reach peak amounts at NF62 and decline towards NF66 (Krain and Denver, 2004). In the lungs and tail, the expression remained low until early prometamorphosis but significantly elevated during climax and remained high until the end (Fig. 1B, C).

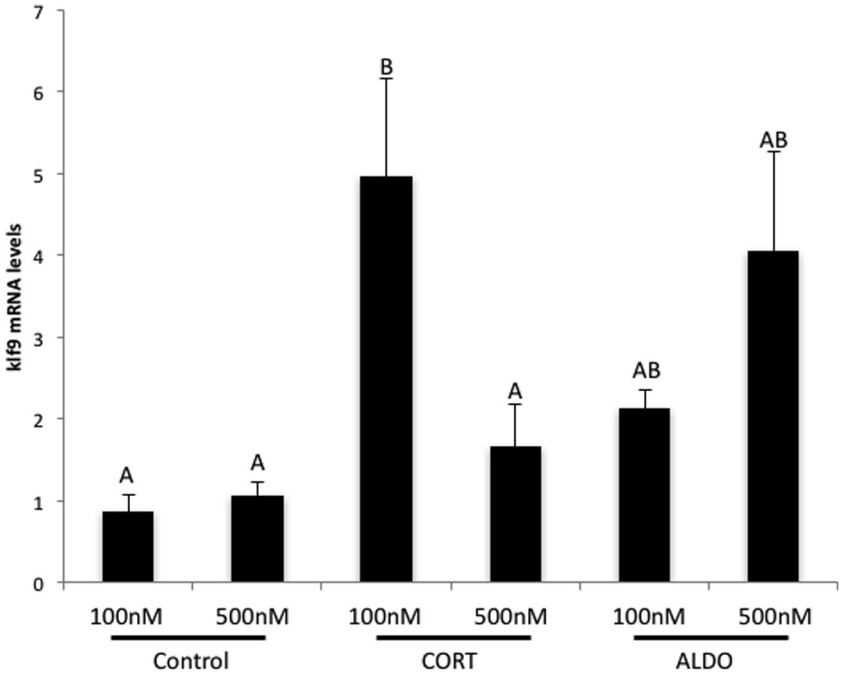

Fig. 3. Dose response of *klf9* expression after CORT and ALDO treatment in tadpole tails. Both CORT and ALDO induced *klf9* expression in the tail but with different dose response curves. Total RNA was extracted from tails harvested from premetmorphic tadpoles (NF54) treated with 100 and 500 nM of CORT and ALDO or vehicle controls for 24 h to measure *Klf9* mRNA expression by quantitative PCR. Bars show mean mRNA levels relative to the reference gene *rpL8*. Error bars indicate SEM. The letters above the error bars indicate significance groups among treatments based on Tukey's honest significant difference test ($p < 0.05$, n = 5 for brain and tails and n = 3 for lungs per treatment group).

It is not known if *klf9* levels remain elevated indefinitely in lungs in juveniles and adults.

3.2. Developmental expression profile of type-I and type-II receptors

The expression of type-I and/or type-II receptor is likely a prerequisite for tissues to express *klf9* in response to corticosteroids. We thus compared the developmental expression of both corticosteroid receptors in the brain, lungs, and tail of *X. tropicalis* tadpoles from premetamorphosis to the end of climax (Fig. 2). For the type-I receptor, the expression levels in the brain, lungs, and tail all increased significantly reaching a peak at the climax of metamorphosis. As seen for the type-I receptor, the type-II mRNA in the lungs showed significant increases and remained elevated at NF66. However, type-II receptor expression in brain and tail, though present, did not change significantly across metamorphosis.

3.3. Dose response for CORT and ALDO on klf9 expression

Because both type-I and type-II receptors are expressed in each tissue throughout metamorphosis, we wanted to further examine the potential role of these receptors in *klf9* expression. Dose response and time course experiments were carried out first to establish suitable treatments for our receptor antagonist experiments in the next section. In the dose response experiments (Fig. 3), CORT-induced *klf9* expression occurred at 100 nM but was not significant at 500 nM consistent with an inverted U-shaped dose response or indicative of more rapid transient expression kinetics at 500 nM. However, for ALDO from 100 nM to 500 nM, *klf9* mRNA levels increased in a dose-dependent manner (Fig. 3). For the time course experiment (Fig. 4), *klf9* expression increased significantly in each tissue reaching highest levels at 4 h in brain and lungs and at 24 h in tail. Thus, we used 100 nM CORT and 500 nM ALDO at a 4-h time point for the brain and lungs and a 24-h time point for the tail in the following experiments.

3.4. Effect of receptor antagonists on corticosteroid-induced kfl9 expression

To examine the *in-vivo* receptor(s) used by corticosteroids, we studied the effect of RU486 (type-II antagonist) and spironolactone (SL, type-I antagonist) on *klf9* expression induced by CORT (Fig. 5) and ALDO (Fig. 6). Addition of receptor antagonists alone had no impact on *klf9* regulation *in-vivo*, but RU486 blocked the induction of *klf9* by CORT completely in all three tissues, while addition of SL had no effect on CORT-induced *klf9* induction (Fig. 5). Similarly, ALDO-induced *klf9* expression was completely blocked by RU486 while SL had no effect (Fig. 6).

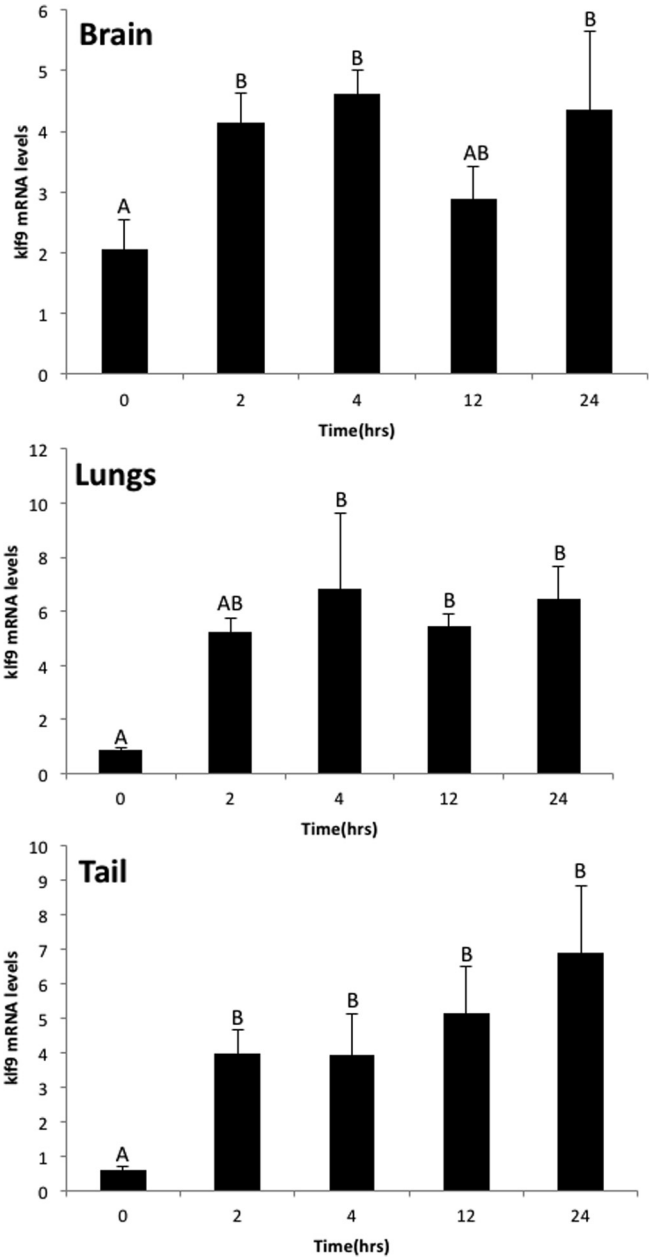

Fig. 4. Time course profile of *klf9* induction by CORT for brain, lungs, and tail. The expression of *klf9* peaked at 4 h in brain and lungs and at 24 h in the tail. Total RNA was extracted from tissues harvested from premetmorphic tadpoles (NF54) treated with 100 CORT or vehicle controls at the indicated time points to measure *Klf9* mRNA expression by quantitative PCR. Bars show mean mRNA levels relative to the reference gene *rpL8*. Error bars indicate SEM. The letters above the error bars indicate significance groups among treatments based on Tukey's honest significant difference test ($p < 0.05$, $n = 5$ tails per treatment group).

4. Discussion

Stress has numerous pleiotropic actions on organ maturation and development as mediated by corticosteroid hormones (Fowden and Forhead, 2015). While corticosteroid signaling is vital for maturation of major organ systems at the end of the gestational period, the endogenous roles of these hormones, their receptor preferences and subsequent gene targets are yet to be established. Here, we chose to address this issue by examining the endogenous regulation of the only known CORT-response gene in tadpoles, *klf9* by CORT, ALDO, and their receptors in three corticosteroid-responsive tissues *in-vivo* in tadpoles.

We found that expression of *klf9* starts low and by metamorphic climax achieves a 10-, 20-, and 100-fold increase in brain, lungs, and tail, respectively. These data concur with previous studies in brain and tail and add lung as another tissue with dynamic *klf9* expression (Hoopfer et al., 2002). Similarly, our measurements for both corticosteroid hormone receptors showing expression in brain, lungs, and tail and increases in expression across development in the case of the type-I receptor for each tissue and only in the lung for the type-II receptor are in line with previous studies in brain for type-II receptor (Krain and Denver, 2004) and are novel for the lung and for the type-I receptor in brain, tail, and lung. For tail type-II expression, we found no significant differences among stages, though there was a 50% increase from NF54 to NF64, compared to a threefold increase in type-II receptor in the tail from a previous study using Northern blot analysis (Krain and Denver,

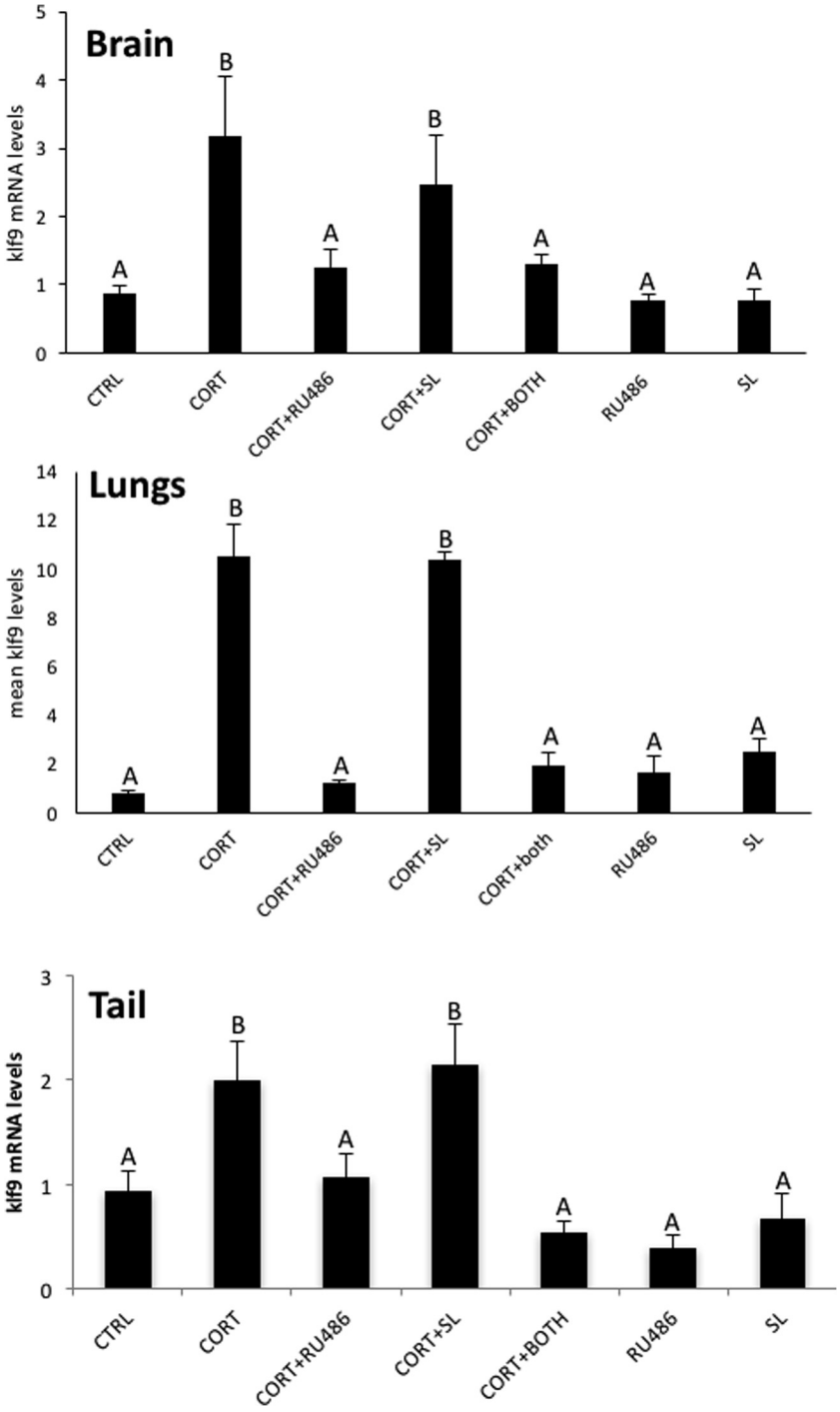

Fig. 5. Effect of receptor antagonists on CORT-induced *klf9* expression in brain, lungs, and tail. CORT-induced *klf9* expression was completely suppressed by type-II receptor antagonist but not by type-I receptor antagonist in each tissue. Tadpoles were raised to NF54 and treated with 100 nM CORT, 150 nM RU486, 150 nM spironolactone (SL), and EtOH control as indicated before tissue harvest at 4 h (brain and lungs) and 24 h (tails). Total RNA was isolated from brain, lungs, and tail, and *klf9* mRNA levels were measured using quantitative PCR and normalized to the reference gene *rpL8*. Error bars indicate SEM. The letters above the error bars indicate significance groups among treatments based on Tukey's honest significant difference test ($p < 0.05$, $n = 5$ for brain and tails and $n = 3$ for lungs per treatment group).

2004). Thus, both corticosteroid hormone receptors are present and available to regulate *klf9*. In addition, previous studies showing significant increases in CORT and ALDO levels (Carstensen et al., 1961; Jolivet Jaudet and Leloup Hatey, 1984) and our current results and results from others on dynamic type-I and type-II receptor expression during development (Fig. 2) (Funder, 1997; Langlois and Martyniuk, 2013; Roubos et al., 2009) strongly suggest developmental roles each corticosteroid hormone and receptor.

To identify potential functional roles of corticosteroid signaling *in-vivo*, we examined which corticosteroid(s) and receptor(s) are able to regulate *klf9* in different tissues. Our dose response study using premetamorphic tadpoles confirmed *klf9* induction in tails by CORT (Bonett et al., 2009) and showed ALDO was also capable of inducing *klf9* but at a higher concentration (500 nM). Receptor antagonist studies showed that RU486 (type-II receptor-specific antagonist) was able to completely block CORT induction of *klf9*.

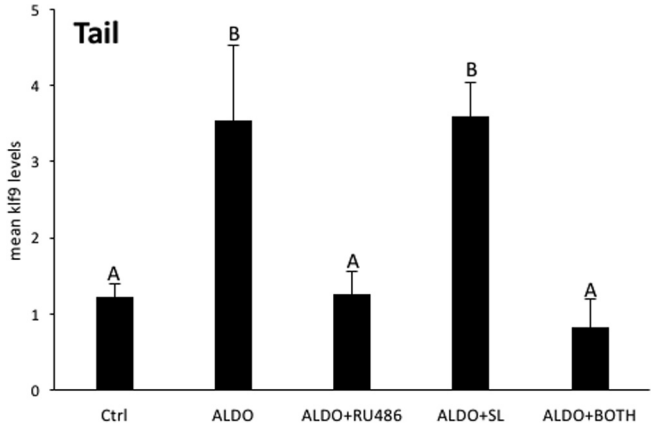

Fig. 6. Effect of receptor antagonists on ALDO-induced *klf9* expression in tails. As for CORT, ALDO-induced *klf9* expression was completely suppressed by type-II receptor antagonist but not by type-I receptor antagonist. Tadpoles were raised to NF54 and treated with 100 nM ALDO, 150 nM RU486, 150 nM spironolactone (SL), and EtOH control as indicated before tail harvest at 24 h. Total RNA was isolated from tails, and *klf9* mRNA levels were measured using quantitative PCR and normalized to the reference gene *rpL8*. Error bars indicate SEM. The letters above the error bars indicate significance groups among treatments based on Tukey's honest significant difference test ($p < 0.05$, $n = 5$ tails per treatment group).

If the type-I receptor played a role, one would expect RU486 to only partially block *klf9* induction. Our result using RU486 is consistent with the result from spironolactone (type-I receptor-specific antagonist), which did not block *klf9* induction, indicating that even though type-I receptor is present, CORT seems to use only the type-II receptor in *klf9* regulation.

Similar to the results with CORT, ALDO is also able to induce *klf9*, but the higher dose required, 500 nM, represents supraphysiological levels which may have been sufficient to activate the type-II receptor. Indeed, we found that RU486 and not SL blocked ALDO induction of *klf9*. Thus, ALDO induction of klf9 does not involve the type-I receptor. Also, because the low affinity of ALDO for the type-II receptor likely precludes its use *in-vivo* by endogenous levels of ALDO, we conclude that ALDO induction of *klf9* does not occur *in-vivo*.

Our findings expand on results from studies of postnatal mice, juvenile *Xenopus*, and cell culture experiments that CORT acts through type-II receptors and is the ligand/receptor pair that carries out the effects of corticosteroids on *klf9* regulation (Bagamasbad et al., 2012; Bonett et al., 2010; Terrien and Prunet, 2013). Our studies focused on a single corticosteroid-response gene, *klf9*. Whether this result will hold for other CORT-response genes or for the effects of stress on development in general remains to be examined. Increased understanding would proceed from measuring ALDO levels in response to stress in tadpoles or using knockout hormone/receptor frog lines to assess the developmental roles of these hormones during metamorphosis. In addition, the peak in ALDO levels at metamorphic climax and the dynamic developmental expression profile of the type-I receptor in all three tissues highlights additional unanswered questions in the role of corticosteroids and their receptors during development.

Disclosure statement

The authors have nothing to disclose.

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