General and Comparative Endocrinology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

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*

ARTICLE INFO

*Article history:*
Received 19 November 2016
Accepted 17 February 2017
Available online xxxx

*Keywords:*
Amphibian metamorphosis
Corticosterone
Aldosterone
Glucocorticoid receptor
Mineralocorticoid receptor
RU486
Spironolactone

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.

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## 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).

http://dx.doi.org/10.1016/j.ygcen.2017.02.007
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Please cite this article in press as: Shewade, L.H., et al. *In-vivo* regulation of Krüppel-like factor 9 by corticosteroids and their receptors across tissues in tadpoles of *Xenopus tropicalis*. Gen. Comp. Endocrinol. (2017), http://dx.doi.org/10.1016/j.ygcen.2017.02.007

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

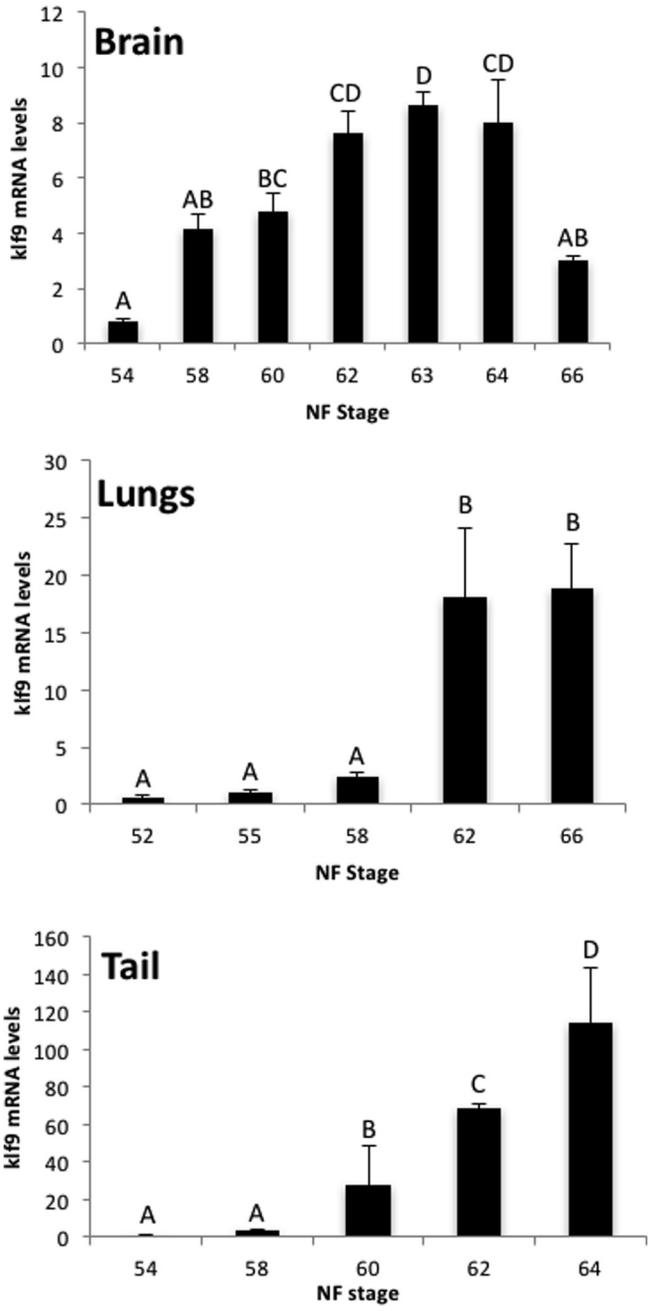


**Fig. 1.** Developmental profile of *klf9* expression in the brain, lungs, and tail during *Xenopus* metamorphosis. The expression of *klf9* increased significantly during metamorphosis in each tissue. Total RNA was collected from tadpole tissues at the indicated developmental stages to measure *klf9* 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).

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