ABSTRACT

Title of Thesis:	PITUITARY-TARGETED KNOCKOUT OF GLUCOCORTICOID RECEPTORS SUPPRESSES GROWTH HORMONE EXPRESSION DURING MOUSE EMBRYONIC DEVELOPMENT
	Scott Thomas Klug, Master of Animal Sciences, 2023
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The pituitary, an endocrine gland primarily regulated by the hypothalamus, secretes multiple hormones and regulates the release of several other hormones from multiple glands within the body. Pituitary development is conserved across vertebrate species and involves a complex temporal and spatial balance of multiple transcription and signaling factor gradients, which control cell commitment, differentiation, and proliferation. Proper pituitary development is critical to survival, since several essential physiological processes are regulated by the pituitary during embryogenesis and throughout life, including metabolism, milk production, stress, reproduction, and growth. Glucocorticoids (GCs) produced by the adrenal glands during embryogenesis play an important role in the differentiation of somatotrophs, the endocrine cell type within the pituitary that produces growth hormone (GH), as well as *GH* mRNA expression in both avian and mammalian species. In chickens, somatotrophs differentiate between e12 and e16, while mouse somatotrophs differentiate between e15.5 and e17.5. The establishment of the

hypothalamic-pituitary-adrenal (HPA) axis and a rise in circulating levels of corticosterone (CORT), the primary GC in rodents and chickens, coincides with somatotroph differentiation and *GH* mRNA expression in both rodents and chickens. Furthermore, exogenous synthetic GCs such as dexamethasone have been shown to prematurely differentiate somatotrophs in rats and chickens *in vitro* and *in vivo*. GCs generate physiological reactions by binding to target cells that express the glucocorticoid receptor (GR). GR, in turn, acts as a transcription factor and regulates the expression of several genes. Despite numerous studies on *GH* mRNA regulation and somatotroph differentiation by GCs, the mechanism is not completely understood. To better understand the role GR plays in GH regulation by GCs in a mammalian model, mouse embryos with pituitary-targeted GR knockout were generated utilizing the Cre-LoxP Recombinase system under control of the pituitary-specific *αGSU* promoter. *GH* mRNA was significantly decreased in $GR^{(-/-)}$ embryos, while $GR^{(+/-)}$ embryos expressed intermediate levels of *GH* mRNA in comparison to wild-type $GR^{(+/+)}$ embryos. Significant differences in expression of other pituitary hormones in $GR^{(-/-)}$ embryos were not observed, indicating that GR must not play an essential role in regulating the expression of any other pituitary hormone gene. Furthermore, all $GR^{(-/-)}$ embryos died at birth, or soon after. To our knowledge, this is the first study to report homozygous GR knockout in the pituitary suppresses embryonic GH expression and results in a neonatal lethal phenotype.

PITUITARY-TARGETED KNOCKOUT OF GLUCOCORTICOID RECEPTORS SUPPRESSES GROWTH HORMONE EXPRESSION DURING MOUSE EMBRYONIC DEVELOPMENT

by

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In loving memory of

Caleb McCurdy

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List of Abbreviations

ACTH	Adrenocorticotrophic hormone
ADH	Antidiuretic hormone
ANOVA	Analysis of variance
BMPs	Bone morphogenetic proteins
BSA	Bovine serum albumen
cDNA	Complementary deoxyribonucleic acid
CORT	Corticosterone
CRE	Cre recombinase enzyme
CRH	Corticotropin-releasing hormone
DHH	Desert Hedgehog
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
DTT	Dithiothreitol
e	Embryonic day
FGFs	Fibroblast growth factors
FLOX	Flanking loxp sequences
FSH	Follicle-stimulating hormone
FSHβ	Follicle-stimulating hormone beta subunit
GCs	Glucocorticoids
GH	Growth hormone
GHRH	Growth hormone-releasing hormone
GR	Glucocorticoid receptor
GREs	Glucocorticoid response elements
Hes	Hairy enhancer of Split
HH	Hedgehog
HPA	Hypothalamic-pituitary-adrenal
ICC	Immunocytochemistry
IGF-1	Insulin-like growth factor 1
IHH	Indian Hedgehog
ISLET1	Insulin gene enhancer protein ISL-1
LBD	Ligand-binding domain
LH	Luteinizing hormone
LHβ	Luteinizing hormone beta subunit
MEM	Minimum essential medium
NICD	Notch intracellular domain
NLS	Nuclear localization signal
NTD	N-terminal domain
OT	Oxytocin
PIT1	Pituitary-specific transcription factor 1
POMC	Proopiomelanocortin
PRL	Prolactin
PROP1	Prophet of Pit1

RA	Retinoic Acid
RARA	Retinoic acid receptor antagonist
RHPA	Reverse hemolytic plaque assay
RNA	Ribonucleic acid
RP	Rathke's pouch
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
RX/RAX	Retina and anterior neural fold homeobox protein
SAG	Smoothened Agonist
SF1	Steroidogenic factor 1
SHH	Sonic Hedgehog
SMO	Smoothened
SST	Somatostatin
T-RNA	Total RNA
TSH	Thyroid-stimulating hormone
TSHβ	Thyroid-stimulating hormone beta subunit
WNT	Wingless-related integration site
WT	Wild type
αGSU	alpha glycoprotein subunit

Chapter 1: Literature Review

Introduction

The pituitary gland

The pituitary, or hypophysis, is an endocrine gland primarily regulated by the hypothalamus, which secretes multiple hormones and regulates the secretion of several other hormones from other glands within the body. The hypophysis is divided into two functionally and anatomically distinct regions, the adenohypophysis, or anterior pituitary, and the neurohypophysis, or posterior pituitary. The anterior pituitary consists of five unique endocrine cell types, including somatotrophs, lactotrophs, gonadotrophs, thyrotrophs, and corticotrophs, which release growth hormone (GH), prolactin (PRL), luteinizing hormone (LH) and follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), and adrenocorticotrophic hormone (ACTH), respectively. The posterior pituitary is an extension of the hypothalamus, consisting of neuronal cell bodies located within the hypothalamus that synthesize oxytocin (OT) and antidiuretic hormone (ADH), which are then released into the circulation from neuronal terminals located in the posterior pituitary. When the hypothalamus receives external or internal stimuli, it releases signaling factors into short portal vessels leading to the anterior pituitary that either stimulate or inhibit the release of hormones from the anterior pituitary gland. When the pituitary is stimulated, it releases hormones into the circulatory system that bind to receptors on target endocrine glands to regulate several essential physiological processes, including reproduction, milk production, osmotic balance, stress, metabolism, and growth (Treier and Rosenfeld, 1996).

Glucocorticoids

Glucocorticoids (GCs) are endogenous steroid hormones released from the cortex of the adrenal gland which are essential to life. They are critical during embryogenesis, as well as throughout life, in the regulation of multiple important physiological processes, including the response to stress, the immune system, metabolism homeostasis, and the development of multiple tissues (Proszkowiec-Weglarz and Porter, 2010; Busada and Cidlowski, 2017). In the pituitary gland, GCs are crucial to the differentiation and production of growth hormone (GH) from somatotrophs (Fu and Porter, 2004; Bossis and Porter, 2003; Porter et al., 2001; Bossis and Porter, 2000; Ellestad et al., 2009). The development of the pituitary is conserved across all vertebrates, and extensive work has been conducted on the effects of glucocorticoids during embryogenesis on somatotroph differentiation, as well as the regulation of GH expression. While much progress has been made in elucidating the role of glucocorticoids in somatotroph differentiation and GH regulation, the mechanisms are not completely clear.

Pituitary development

The pituitary develops during embryogenesis in conjunction with the hypothalamus. Direct contact between the ectoderm layers that ultimately develop into the pituitary and hypothalamus is required during development for both tissues to be established, as well as postnatally for proper communication. In the past 30 years, a significant amount of research has been conducted, mainly on mice with spontaneous or genetically edited mutations, to study genes involved in the development of the pituitary (Xatzipsalti et al., 2019). Fate map studies in chicken

and zebrafish embryos have demonstrated that pituitary development is highly conserved in vertebrates. This has led to the identification of mutations in several genes that encode transcription and signaling factors that are associated with human pituitary disorders (Kelberman et al., 2009). The development of the pituitary during embryogenesis is orchestrated through a temporal and spatial balance of various transcription and signaling factor gradients, which influence cellular differentiation and proliferation (Vakili and Cattini, 2012).

The pituitary gland is located within a recess in the sphenoid bone, known as the *sella turcica*, at the base of the brain. A mature pituitary consists of anterior and posterior regions because each region is derived from separate embryonic ectoderm layers. Upon head folding, oral ectoderm invaginates cranially and comes into contact with the neural ectoderm of the ventral diencephalon. This invagination, known as Rathke's pouch (RP), is the precursor structure to the anterior pituitary (Watanabe, 1982; Rosenfeld et al., 1999). Soon after RP is visible, the neural ectoderm of the ventral diencephalon evaginates caudally toward the oral ectoderm layer, forming a rudimentary posterior pituitary. The direct contact and cell signaling that occurs between the oral and neural ectoderm layers that eventually form the mature pituitary gland are essential for proper function and development.

Rathke's pouch development

RP development overlaps with the development of the posterior pituitary and can be divided into four stages (Xatzipsalti et al., 2019). In mice, the first stage of pituitary development takes place 7.5 days post coitum (dpc), when oral ectoderm thickens and forms the hypophyseal placode, anterior to the future ventral

diencephalon. The second stage begins by 9 dpc when the hypophyseal placode invaginates dorsally to begin forming a rudimentary Rathke's pouch, contacting a region of the ventral diencephalon known as the infundibulum. By 10 dpc, the infundibulum begins to evaginate towards RP, and eventually develops into the posterior pituitary. The third stage of RP development occurs by 12.5 dpc, when RP, through a series of proliferation and apoptosis events, separates from the original oral ectoderm it is derived from and becomes a definitive pouch. Interestingly, the lumen of RP persists during development in rodents, dividing the anterior and posterior pituitary lobes, forming an intermediate lobe which contains melanotrophs. In humans, the intermediate lobe is residual. The final stage, occurring between 12.5 and 17.5 dpc, is defined by the proliferation of progenitor cells and terminal differentiation of all endocrine cell types (Xatzipsalti et al., 2019). Each endocrine cell population differentiates in a temporally conserved manner across all species, with differentiation defined as when the expression of the secreted hormone is observed. Interestingly, birth dating studies in mice have shown that between 11.5 and 13.5 dpc, the majority of anterior pituitary endocrine cell types have exited the cell cycle, indicating endocrine cells can commit to their specific cell type before they terminally differentiate and begin to express hormones (Davis et al., 2011).

The development of RP follows the same four stages in avian species as it does in rodents. In the chicken, oral ectoderm thickening can be visualized by stage HH10 (33-38 hours of incubation) (Sánchez-Arrones et al., 2015; Proszkowiec-Weglarz et al., 2011). Around stage HH12, or 45-49 hours of incubation, oral ectoderm starts to invaginate to form an observable RP. Around HH29 (6 days of

incubation), RP loses contact with the oral ectoderm and begins to proliferate. The neuroectoderm of the infundibulum evaginates toward RP to form a rudimentary posterior pituitary between stages HH29-30 (6-7 days of incubation) (Sánchez-Arrones et al., 2015; Proszkowiec-Weglarz et al., 2011). Figure 1 provides a schematic summary of pituitary formation during mouse embryonic development (taken directly from Rizzoti K., 2015).

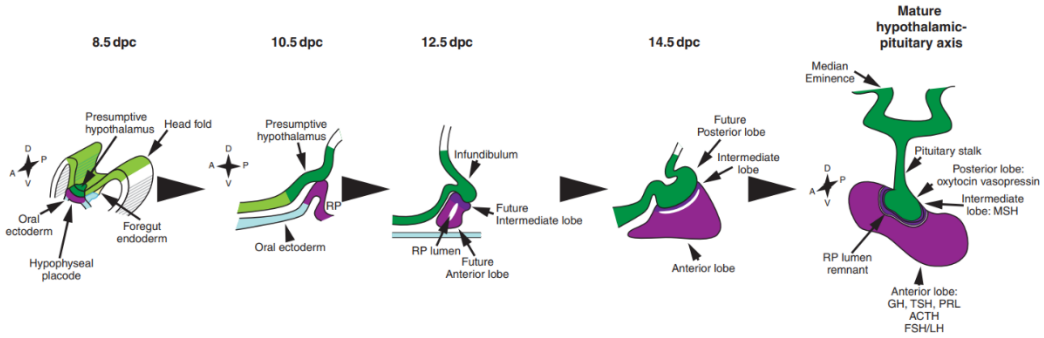


FIG 1. A schematic summary of pituitary formation during embryonic mouse development. By 8.5 dpc, oral ectoderm makes physical contact with neural ectoderm and begins to thicken to form the hypophyseal placode. Between 10.5 -12.5 dpc, oral ectoderm invaginates cranially toward the neural ectoderm layer, while neural ectoderm evaginates caudally toward the oral ectoderm. Through a series of proliferative and apoptotic events, the oral ectoderm that invaginated loses contact from the original layer it is derived from. This represents the future anterior lobe of the pituitary. By 14.5 dpc, the posterior lobe has formed, and a mature pituitary gland is established soon after (taken directly from Rizzoti K., 2015).

Signaling and Transcription Factors involved in Pituitary Development

Signaling Pathways

In both rodents and birds, it is clear that each stage of pituitary development is tightly regulated by signaling pathways that coordinate the expression of transcription factors, which in turn direct RP progenitor cells to proliferate, commit to specific hormone-producing cell types, and terminally differentiate (Dasen and Rosenfeld, 1999). These signaling pathways involve signaling molecules that influence RP development, and ultimately formation of the anterior pituitary. The majority of these signaling molecules are produced and released from the ventral diencephalon, including bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs). However, some of these signaling molecules are present in both RP and the ventral diencephalon, such as Sonic hedgehog (SHH), WNT/β-catenin, and Notch signaling. Figure 2 is a schematic summary of signaling molecules, transcription factors, and cell lineages throughout mouse embryonic pituitary development (taken directly from Kelberman et al., 2009).

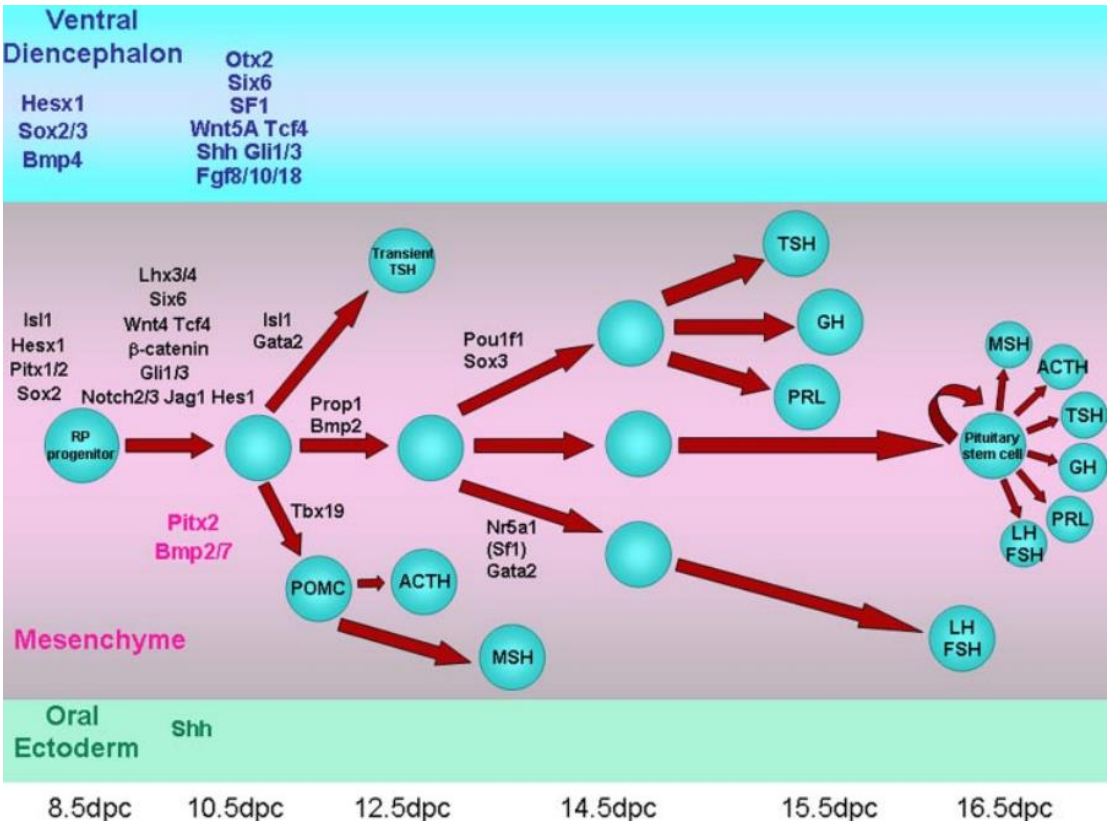


FIG 2. Schematic of the signaling pathways and transcription factors that regulate the commitment and differentiation of endocrine cells during embryonic mouse pituitary development (taken directly from Kelberman et al., 2009).

WNT/β-catenin signaling

Wingless-related integration site (WNT) signaling molecules are largely known for their role in cell proliferation, determination, and differentiation events during embryogenesis, as well as in adulthood. WNT signaling is required in the ventral diencephalon and RP, and there are three known pathways. The canonical WNT pathway involves β-catenin, an intracellular protein that is degraded in the absence WNT ligands (Kioussi et al., 2002). Briefly, WNT ligands bind to a complex that consists of Frizzled receptors and LRP proteins. This prevents β-catenin from degrading and allows it to accumulate within the cytoplasm. It then translocates to the nucleus and displaces Groucho, a repressor associated with TCF/LEF transcription factors. WNT target genes, such as *Pitx2*, can now be expressed when Groucho is displaced, leading to progenitor proliferation (see below). The second WNT pathway regulates cell polarity and influences cell morphology and movement, while the third WNT pathway is related to intracellular Ca^{2+} release (Hayward et al., 2008).

TCF4 is a downstream effector of WNT signaling that is present in RP and the ventral diencephalon. *Tcf4* mutants show bifurcations in the developing RP and pituitary dysplasia. Six6 expression is also expanded in these mutants and likely explains this phenotype (Brinkmeier et al., 2003; Li et al., 2002). In the absence of WNT signaling, TCF4 represses SIX6 expression, which in turn represses progenitor cell proliferation. However, when WNT signaling is present, SIX6 expression and proliferation is supported.

Furthermore, *Wnt5a* expression in the ventral diencephalon is considered to ensure correct shaping of RP. Deletion of *Wnt5a* produces a misshaped RP, with extra

bifurcations (Cha et al., 2004). The misshaping of RP is thought to be a result of the disrupting of the ventral boundary of FGFs and BMP4 expression at 10.5 dpc in the infundibulum (Brinkmeier et al., 2007). Additionally, WNT signaling appears to have crosstalk with the Notch signaling pathway, adding complexity to understanding the direct effects of WNT/β-catenin signaling on the development of RP and the ventral diencephalon (Hayward et al., 2008).

Notch signaling

Notch proteins are transmembrane receptors that contain extracellular and intracellular domains. Mammals contain four notch receptors, including Notch1, Notch2, Notch3, and Notch4, and five ligands, Jagged1, Jagged2, Delta1, Delta3, and Delta4 (Baron, 2003). When a notch ligand binds and activates a notch receptor, the notch intracellular domain (NICD) is cleaved and translocates to the nucleus to form a complex with the RBP-J/CSL protein. Repressors are displaced by this complex, allowing target genes of Notch signaling to be activated. These target genes transcribe other transcription factors that control cell fate decisions, including members of the Hairy enhancer of Split (Hes) and bHLH (Hayward et al., 2008). Notch signaling in the ventral diencephalon is not completely understood, but it is known to be required for the proper morphogenesis of the infundibulum (Zhu et al., 2006). In RP however, multiple Notch signaling members, including Notch2, Notch3, Jagged1, and Hes1, are expressed at 9.5 dpc. They are soon downregulated, but expression is maintained in the lumen of RP during development (Raetzman et al., 2004). Interestingly, Notch signaling is observed in the adult pituitary in the same locations where pituitary stem cells reside, indicating a role in the maintenance of stem cells (Chen et al., 2006).

When Rbp-J was deleted in RP, premature differentiation of corticotrophs was observed. *Hes1*$^{(-/-)}$ embryos also displayed this phenotype, and the authors associated it with decreased proliferation. Furthermore, *Prop1*, a direct target of RBP-J, fails to be expressed at 12.5 dpc in *Rbp-J*$^{(-/-)}$ embryos, resulting in a lack of *Pit1* expression (Zhu et al., 2006; Kita et al., 2007). Therefore, Notch signaling is thought to be required to regulate differentiation, promote proliferation, and maintain undifferentiated progenitors.

Bone Morphogenetic Proteins and Fibroblast Growth Factors

Bone Morphogenetic Proteins (BMPs) are secreted ligands that bind to serine-threonine receptor kinases and transduce intracellular cascades that regulate transcription of different genes. BMP4 is required for RP formation and maintenance. Expression of BMP4 is first seen within the developing infundibulum at 8.5 dpc, where it is maintained until 14.5 dpc. It is the earliest signaling molecule to be expressed in the infundibulum (Ericson et al., 1998). *Bmp4*$^{(-/-)}$ embryos die during development, and do not form RP (Winnier et al., 1995). Furthermore, deleting the BMP receptor gene *Bmpr1a*, within RP on 9.5 dpc results in an undeveloped RP on 10.5 dpc (Davis and Camper, 2007). Additionally, BMP4 regulates *Isl1* expression (see below). *Isl1* and *Bmp4* expression is correlated, with upregulation and downregulation of each gene following the same pattern (Treier et al., 1998). In summary, BMP4 is required for RP formation and maintenance, in part by regulating *Isl1* expression.

Fibroblast growth factors (FGFs) are secreting signaling molecules that bind receptor tyrosine kinases and activate several signaling cascades that influence

cellular proliferation, maintenance, and motility during embryogenesis. *Fgf8*, *Fgf10*, and *Fgf18* are expressed in the infundibulum 24 hours after *Bmp4* expression is first observed (Ericson et al., 1998). FGFs interact with BMPs to create restricted domains of proliferating and differentiating cells and control the timing of these events by the genes they regulate. In the chick, the development and expansion of the infundibulum required FGF expression (Pearson et al., 2011). In mice, FGFs are required in RP dorsally to inhibit differentiation by downregulating *Isl1*, creating a domain of proliferation ventrally. FGFs also induces *Lhx3* and *Lhx4* in RP, which maintains proliferation of progenitors (Davis et al., 2011).

Sonic Hedgehog Signaling

Three hedgehog (HH) proteins are known to exist in mammals, Indian Hedgehog (IHH), Desert Hedgehog (DHH), and Sonic Hedgehog (SHH). Each of these proteins act as morphogens in different tissues during development and evoke different cellular responses. HH signaling involves the receptor Patched, the transmembrane protein smoothened (SMO), and GLI transcription factors (Carballo et al., 2018). When SHH is not expressed, the receptor Patched inhibits SMO. When SHH is expressed, it binds to Patched, which releases SMO. SMO then activates GLI transcription factors which can repress or activate other genes. Sonic Hedgehog (*Shh*) is expressed in the ventral diencephalon and oral ectoderm; however, expression is lost in RP as it forms, and by 12 dpc, it is completely lost throughout all of the oral ectoderm (Treier et al., 2001). Patched receptors and GLI transcription factors, however, are still expressed in oral ectoderm and RP, indicating that SHH influences its formation. By 14 dpc, *Shh* expression is also lost in the ventral diencephalon

(Treier et al., 2001). In the absence of SHH within the ventral diencephalon, *Bmp4* expression is expanded ventrally, and RP forms twice (Trowe et al., 2013). Therefore, SHH seems to play an important role in the ventral diencephalon by antagonizing BMP signaling to ensure proper morphogenesis, which in turn ensure proper RP development.

Transcription factors

Several transcription factors, regulated by the previously mentioned signaling pathways, have been identified to be essential to RP development, including *Isl1*, *Lhx3*, *Lhx4*, *Pitx1*, *Pitx2*, *Hesx1*, *Sox2*, *Sox3*, *Rx/Rax*, *Six3*, and *Six6*. Other transcription factors, such as *Prop1*, *Pit1*, *Gata2*, *Gata3*, *Nr5a1*, *and Tbx19* are required for progenitor cell specification and differentiation (Kelberman et al., 2009). Some of these transcription factors are exclusively found in the ventral diencephalon and play a role in the morphogenesis of the infundibulum, which can indirectly affect pituitary and RP morphogenesis. Others are present in both the ventral diencephalon and RP, while some are present in RP alone.

Isl1

Insulin gene enhancer protein ISL-1 (ISLET1) is a LIM-homeodomain transcription factor that is essential for cell fate decisions in various embryonic stem cells. In regard to the pituitary, ISL1 is expressed in oral ectoderm on 8.5 dpc, as well as RP on 9.5 dpc. Between 10.5 and 11.5 dpc, expression of ISL1 becomes restricted to prospective thyrotrophs in the ventral region of the rostral tip of RP (Ericson et al., 1998). Interestingly, ISL1 expression is maintained in gonadotrophs in the adult

pituitary (Schang et al., 2013). Furthermore, ISL1 has been suggested to be regulated by BMPs, and to participate in Lhx3 activation (Davis and Camper, 2007; Mullen et al., 2012). *Isl1* null mice display a hypoplastic RP and die on 10 dpc, indicating that ISL1 may be required for RP progenitor cell viability and maintenance (Takuma et al., 1998).

Lhx2, Lhx3, and Lhx4

LHX2, LHX3, and LHX4 are more examples of transcription factors that belong to the LIM-homeodomain family. *Lhx2* expression is observed in the ventral diencephalon during RP development, and evagination and proliferation of the infundibulum does not occur in *Lhx2*$^{(-/-)}$ mice (Zhao et al., 2010). Furthermore, BMP and FGF signaling expression in these mice is expanded ventrally, resulting in RP to develop more rostrally rather than dorsally. Interestingly, all endocrine cell types are observed in these mice, indicating that cell differentiation is not altered in *Lhx2*$^{(-/-)}$ mice (Zhao et al., 2010).

LHX3 and LHX4 are both required for proper pituitary development in mice, and mutations in *Lhx3* and *Lhx4* have been identified in humans with combined pituitary hormone deficiency (Sheng et al., 1997). Both *Lhx3* and *Lhx4* are expressed in RP by 9.5 dpc, however *Lhx3* expression is maintained throughout the adult pituitary, while *Lhx4* expression becomes confined to the anterior lobe and ultimately downregulated by 15.5 dpc (Sheng et al., 1997). *Lhx4*$^{(-/-)}$; *Prop1*$^{(-/-)}$ mice do not express LHX3, indicating that LHX4 and PROP1 may function together to trigger *Lhx3* expression (Raetzman et al., 2002). Both *Lhx4*$^{(-/-)}$ and *Lhx3*$^{(-/-)}$ mice develop hypoplastic pituitaries, however *Lhx3*$^{(-/-)}$ mice have reduced proliferation in all

endocrine cell types, while *Lhx4*$^{(-/-)}$ mice are composed of all endocrine cell lineages (Prince et al., 2011). Furthermore, NOTCH2, which is normally expressed in the pituitary at the border of dorsal proliferation and ventral differentiation patterning, is not expressed in *Lhx3*$^{(-/-)}$ mice (Ellsworth et al., 2008). In summary, LHX2, LHX3, LHX4 are all required for proper RP and pituitary development. LHX2 indirectly alters RP development by disrupting ventral diencephalon development. LHX3 and LHX4 are both essential during early RP development for progenitor cells to survive, however LHX3 expression is required for endocrine cell line differentiation.

Pitx1 and Pitx2

PITX1 and PITX2 are paired homeodomain transcription factors that are involved in regulating morphogenesis of various organs during development, including RP. They seem to have similar roles in maintaining RP progenitor cells (Charles et al., 2005). Both proteins are expressed during early development in the hypophyseal placode and RP, and expression is maintained in differentiated hormone-producing cells (Lanctot et al., 1997; Gage and Camper, 1997). PITX1 has been shown to interact with PIT1, leading to the activation of *Prl* and *Gh* promoters (Tremblay et al., 1998). Interestingly, *Pitx1*$^{(-/-)}$ mouse mutants die around birth but show normal RP development, while RP stops developing in *Pitx2*$^{(-/-)}$ mice, indicating *Pitx2* may be essential to maintain RP development. Furthermore, *Pitx2* is known to be targeted by Wnt signaling and induce RP progenitor cells to proliferate through direct transcriptional activation of cyclins (Ai et al., 2007; Kioussi et al., 2002). In summary, PITX1 and PITX2 are required for the maintenance of RP development, as well as RP progenitor survival and ultimately endocrine function.

Prop1 and Pou1f1 (Pit1)

Prophet of Pit1 (PROP1), a paired-like homeodomain transcription factor, is the earliest transcription factor to appear that is exclusively expressed in the pituitary, and its expression activates *Pou1f1* (*Pit1*) expression (Olson et al., 2006). PROP1 is first expressed in RP on 10 dpc and levels drastically decrease after peaking on 12 dpc, but constant expression is maintained until 15.5 dpc in the periluminal area in SOX2-expressing progenitors. Ames dwarf mice possess a naturally occurring mutation in the homeodomain of PROP1, reducing its DNA binding activity. *Prop1*$^{(-/-)}$ mice exhibit the same phenotypes as the Ames dwarf mice, including reductions in PIT1 expression and deficiencies in GH, PRL, TSH, and gonadotropins, associated with the loss of the PIT1 cell lineage postnatally (somatotrophs, lactotrophs, thyrotrophs), as well as gonadotrophs (Gage et al., 1996; Andersen et al., 1995; Yoshida et al., 2009). The adult pituitary is hypoplastic in these mice as a result of the failure of dorsal progenitor cells to expand ventrally and differentiate, potentially due to *Notch2* being downregulated (Ward et al., 2006; Himes and Raetzman, 2009). *In vitro* studies have shown that PROP1 forms a complex with β-catenin, and other proteins, that downregulates *Hesx1* expression and activates *Pit1* expression (Olson et al., 2006). The downregulation of *Hesx1*, followed by the upregulation of *Prop1*, represents an important step in pituitary development, shifting from progenitor proliferation and maintenance to progenitor differentiation.

PIT1, a POU homeodomain protein, is exclusively expressed in the pituitary at 13.5 dpc following activation from PROP1. *Pit1* expression peaks by 16 dpc and is maintained in terminally differentiated somatotrophs, thyrotrophs, and lactotrophs

(Rhodes et al., 1994). *Pit1*$^{(-/-)}$ mice display pituitary hypoplasia and have deficiencies in GH, PRL, and TSH postnatally, associated with the loss of somatotrophs, lactotrophs, and thyrotrophs, respectively (Li et al., 1990). These cell types differentiate as usual during embryogenesis, however after birth, proliferation is reduced and they undergo apoptosis, causing hypoplasia (Ward et al., 2006). Therefore, PIT1 is essential for the survival of the somatotrophs, thyrotrophs, and lactotrophs postnatally. Furthermore, PIT1 regulates the timing of differentiation during embryogenesis through interactions with Notch signaling molecules and GATA2, a zinc finger transcription factor that is expressed in thyrotrophs and gonadotrophs. Notch signaling represses the expression of the PIT1-target gene *Math3*. *Math3* encodes the bHLH transcription factor, which is responsible for terminal differentiation of somatotrophs, therefore preventing premature differentiation of somatotrophs (Zhu et al., 2006). Gonadotrophs and thyrotrophs are also regulated by PIT1 interactions with GATA2. GATA2 expression is important for FSH expression in gonadotrophs, and PIT1 has been shown to repress GATA2 expression. However, PIT1 interacts with GATA2 in thyrotrophs to synergistically promote their differentiation by activating the TSHβ promoter (Dasen et al., 1999; Kashiwabara et al., 2009). In summary, PIT1 is required for the survival of somatotrophs, thyrotrophs, and lactotrophs postnatally, and regulates the timing of differentiation during their development.

Hesx1

HESX1, like PROP1, is a paired-like homeodomain transcription factor that is essential for the formation of the infundibulum, and therefore indirectly RP as well.

Hypopituitarism and variable CNS midline defects have been observed in humans with *Hesx1* mutations, and *Hesx1*$^{(-/-)}$ mice display pituitary dysplasia and postnatal lethality (Dattani et al., 1998). HESX1 binds to other proteins to form a complex that represses *Prop1* expression during early pituitary development (Carvalho et al., 2010). HESX1 is initially expressed in developing hypophyseal placode, as well as the rostral neural plate, which later develops into the forebrain and the ventral diencephalon. However, its expression becomes specific to RP progenitors by 9 dpc, where it is maintained until 13.5 dpc (Hermesz et al., 1996). Several other transcription factors activate its expression, including LHX3, GATA2/GATA3, and PITX2 (Olson et al., 2006; Chou et al., 2006). While it is essential to the formation of the forebrain and the ventral diencephalon, it must be downregulated later in development in order for *Prop1* to be expressed. This downregulation is thought to occur through a β-catenin/PROP1 complex (see above). In summary, HESX1 is thought to be essential for correct forebrain morphogenesis, and its downregulation through WNT signaling is required for *Prop1* expression, the PIT1 lineage differentiating, and for proper RP development.

Rx/Rax

The retina and anterior neural fold homeobox protein (RX/RAX) is also a member of the paired-like homeodomain family. It is largely known for its role in eye morphogenesis; however, it is also expressed early in development in the anterior neural plate. Its expression is also maintained in the ventral diencephalon throughout development. By 10.5 dpc in *Rx/Rax*$^{(-/-)}$ mice, the infundibulum fails to evaginate and *Fgf10* is downregulated, leading RP to stop developing past this stage (Medina-

Martinez et al., 2009). Therefore, RX/RAX plays an important role in the development of the infundibulum, which indirectly affects how RP develops.

Six3 and Six6

SIX3 and SIX6 are members of the Sine Oculis homeobox transcription factor family. SIX proteins coordinate proliferation by negatively or positively regulating transcription (Diacou et al., 2018). *Six3* and *Six6* are closely related, and both are expressed in RP and the ventral diencephalon (Larder et al., 2011; Jean et al., 1999). Interestingly, only *Six6* expression is maintained in the adult pituitary, and *Six6*$^{(-/-)}$ mice develop hypomorphic retinas and pituitaries. Additionally, p27KIP1, a cell cycle negative regulator, is downregulated in these mice, providing an explanation to the *Six6*$^{(-/-)}$ mice phenotype (Li et al., 2002). *Six3*$^{(-/-)}$ mice fail to develop RP (Lavado et al., 2008; Andoniadou et al., 2011). Furthermore, *Six3*$^{(+/-)}$; *Hesx1*$^{(+/-)}$ mutants display similar phenotypes to *Hesx1*$^{(-/-)}$ mice, including increased progenitor proliferation and the formation of multiple clefts. These defects are thought to be a result of ectopic WNT/β-catenin activation, indicating SIX3 represses WNT target genes (Gaston-Massuet et al., 2008). In summary, SIX3 represses WNT target genes, while SIX6 promotes RP progenitor cell proliferation by repressing negative regulators of the cell cycle.

Sox2 and Sox3

SOX2 and SOX3 are SOXB1 transcription factors that bind and bend DNA with their HMG domain. Both are found in the ventral diencephalon, however only *Sox2* is expressed in RP (Zhao et al., 2012). In general, they are associated with stem

cells and promote an undifferentiated state. In the ventral diencephalon, they have both been shown to activate Shh, Six3, and Six6 (Zhao et al., 2012; Lee et al., 2012; Lee et al., 2013). In *Sox3*$^{(-/-)}$ embryos, the infundibulum does not completely evaginate, less proliferation is observed, RP is bifurcated, and *Bmp4* and *Fgf8* expression is expanded anteriorly (Rizzoti et al., 2004). As a result, the pituitary in these mice is somewhat hypoplastic, and they display mild hypopituitarism (Kelberman et al., 2006). These phenotypes are thought to be a consequence from the downregulation of *Shh* and *Six6*. SOX2 is required within RP for the proliferation of progenitors.

SOX2 is expressed in early ectoderm, observed in RP on 9.5 dpc, and maintained in adult pituitary stem cells (Fauquier et al. 2008; Andoniadou et al. 2013; Rizzoti et al. 2013). SOX2 is downregulated as differentiation occurs. *Sox2*$^{(-/-)}$ embryos die around implantation, and therefore cannot provide any information on its role in pituitary development. However, when Sox2 is deleted in RP at 12.5 dpc, PROP1 and PIT1 expression is downregulated. As a consequence, proliferation of progenitor cells is reduced in RP, and endocrine cell deficiencies are observed, especially somatotrophs (Jayakody et al., 2012). In summary, SOX2 is required for progenitors to proliferation within RP, and SOX3 is essential for proper evagination of the infundibulum.

Nr5a1 (SF1)

The orphan nuclear receptor 5a1, also known as steroidogenic factor-1 (SF1), is encoded by *Nr5a1*, and is involved in steroid hormone biogenesis. SF1 is expressed throughout the reproductive and adrenal axes during embryogenesis and maintained

postnatally. It is also expressed in the hypothalamus and pituitary and is involved in gonadotroph differentiation by regulating genes that code for LHβ, FSHβ, αGSU, and the GnRH receptor (Barnhart and Mellon, 1994; Brown and Mcneilly, 1997; Nhan et al., 1999). In gonadotroph progenitor cells, SF1 expression is first seen at 13.5 dpc and can be activated by GATA2 expression (Dasen et al., 1999; Ingraham et al., 1994). Pituitary-specific *Nr5a1*$^{(-/-)}$ mice have reduced gonadotropin expression levels and lack secondary sexual characteristics (Zhao et al., 2001). In summary, Nr5a1 is essential for proper gonadotropin expression and gonadotroph differentiation.

Tbx19 (Tpit)

TBX19 (TPIT) is a transcription factor in the T-Box family that is required for the differentiation of corticotrophs and melanotrophs. It is first expressed at 12.5 dpc in the pituitary, where it is maintained in terminally differentiated corticotrophs and melanotrophs. TPIT interacts with PITX1 to regulate *Pomc* expression from corticotrophs (Lamolet et al., 2001; Lavoie et al., 2008). Humans and mice with *Tpit* mutations display dramatic ACTH deficiencies, as well as hypoplastic intermediate lobes. Interestingly, corticotroph commitment is still observed, but the corticotrophs fail to terminally differentiate. Furthermore, Tbx19$^{(-/-)}$ mice have shown to have increased populations of thyrotrophs and gonadotrophs, indicating that TBX19 may play a role in repressing these cell types. The role TBX19 plays in inhibiting gonadotroph and thyrotroph differentiation may be due to how it interacts with SF1 during development (Pulichino et al., 2003a; 2003b). In summary, TBX19 is required for the differentiation and survival of corticotrophs and melanotrophs, and it also plays a role in controlling thyrotroph and gonadotroph differentiation.

Glucocorticoid hormones

Glucocorticoid hormone production from the adrenal cortex is regulated by the hypothalamo-pituitary-axis. When corticotropin-releasing hormone (CRH) is released by the hypothalamus, corticotrophs in the anterior pituitary are stimulated to produce and release adrenocorticotropic hormone (ACTH). ACTH travels through the circulatory system to reach the adrenal glands, stimulating the synthesis of glucocorticoids. In humans, the primary glucocorticoid is cortisol, however birds and rodents primarily produce corticosterone. Glucocorticoids play several different roles throughout the body, including development of tissues, balancing metabolism, and immune system function. The focus of this review is the role of glucocorticoids in somatotroph differentiation and GH expression.

Glucocorticoid receptor and mechanism of action

The glucocorticoid receptor (GR) functions as a ligand-dependent transcription factor and belongs to the nuclear receptor superfamily, consisting of over 150 members. *Nr3c1*, the gene that encodes GR, is located on chromosome 5 in mice and is expressed in almost every tissue throughout the body (Weikum et al., 2017). GR consists of three different functional domains, the N-terminal domain (NTD), the ligand-binding domain (LBD), and the central DNA-binding domain. When ligand is not present, GR is located within the cytoplasm in multiprotein complexes consisting of immunophilins and heat shock proteins. When ligand is present, it binds to GR and the multiprotein complex dissociates, releasing GR. After GR is released from the multiprotein complex, it acts as a transcription factor and regulates gene expression by translocating to the nucleus and binding to

glucocorticoid response elements (GREs) within target genes of GR. Once GR is bound to a GRE, several other co-activator complexes are recruited and bind to the N-terminal domain to promote transcription (Kassel and Herrlich, 2007).

GR is essential to life, and roughly 90% of whole-body glucocorticoid knockout mice (C57/black6) die at birth due to underdeveloped lungs. Fragments of the ligand-binding domain of the glucocorticoid receptor were detected in the mice that survived, however they display severe glucocorticoid resistance and showed an impaired hypothalamo-pituitary-adrenal axis (HPA) with increased levels of *ACTH* mRNA expression and CORT production (Cole et al., 2001). In the chick pituitary, *Nr3c1* mRNA expression is observed by e10 and peaks on e14, coinciding with somatotroph differentiation (Heuck et al., 2009). Many studies have investigated the role of glucocorticoids and GR in somatotroph differentiation and GH expression (see below). In summary, GR functions as a ligand-dependent transcription factor to regulate gene expression and is essential to several physiological processes throughout the body.

Pituitary Somatotrophs

GH secretion from somatotrophs in the pituitary plays a crucial role in regulating embryonic and postnatal growth. Generally, the somatotrophic axis consists of hypothalamic regulators such as somatostatin (SST) and growth hormone-releasing hormone (GHRH), insulin-like growth factor 1 (IGF-1) produced from the liver, and GH from somatotrophs. A considerable amount of research has been conducted into the regulation of GH production during embryogenesis and postnatally because of the importance of GH in human health, as well as meat production in farm animals.

Somatotroph differentiation during embryogenesis

Somatotrophs are derived from the Pit1 lineage, which also includes thyrotrophs and lactotrophs. *Pit1* expression is dependent upon *Prop1* expression, which is first expressed in mouse RP progenitor cells on 10 dpc. On 13.5 dpc, *Pit1* is first expressed within the pituitary, where it is maintained in somatotrophs throughout adulthood (Bodner et al., 1988; Simmons et al., 1990). By 15.5 dpc, somatotrophs are considered differentiated when GH expression is first observed within the pituitary, however it is not until 17.5 dpc that populations of somatotrophs resemble the numbers within an adult mouse pituitary (Japon et al., 1994).

Prop1 is also required for *Pit1* expression in chickens. While *Pit1* is first observed on e5 in chickens, GH expression is not observed until e12, and significant somatotroph populations are established by e16 (Van et al., 2000; Porter et al., 1995). *Pit1* is often considered to be required for somatotroph differentiation, but somatotrophs have been shown to differentiate in mice during embryogenesis without

Pit1 expression. However, the number of somatotrophs is severely reduced. They also fail to be maintained postnatally, leading to a hypoplastic gland. This indicates that PIT1 is essential to the maintenance of somatotrophs, as it is expressed throughout adulthood in somatotroph populations (Ward et al., 2006).

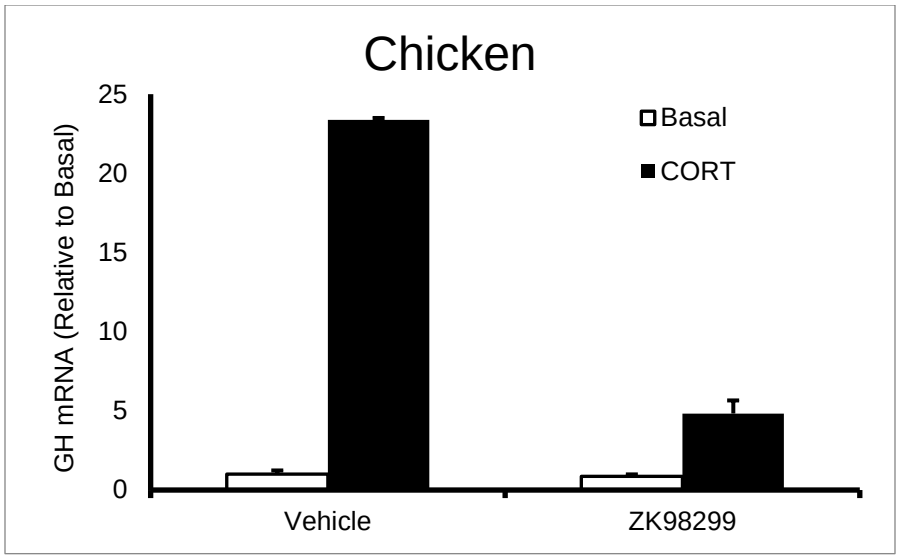
PIT1 is essential to maintaining somatotroph populations in part because it acts as a transcription factor that regulates GH expression by binding to sequences on the GH promoter. PIT1 contains an N-terminal transactivation domain, as well as a C-terminal POU domain which is associated with DNA binding. Variations of PIT1 exist between species as a result of splicing, translation, or alternative transcription events, and each of these variants has varying abilities to regulate GH expression. In the chicken, PIT1α, PITβ1, and PITβ2 all have the ability to activate the GH promoter (Porter and Mukherjee, 2012).

Several examples of mutant mice with spontaneous mutations in both Pit1 and Prop1 have been identified. The Ames dwarf mouse has two mutated copies of *Prop1*. As a result, they have hypoplastic pituitaries and very low numbers of lactotrophs, thyrotrophs, and somatotrophs. The Snell dwarf mouse has a point mutation in Pit1 that alters its DNA binding activity. These mice also have hypoplastic pituitaries, however they have no expression of GH, TSH, and PRL (Li et al., 1990).

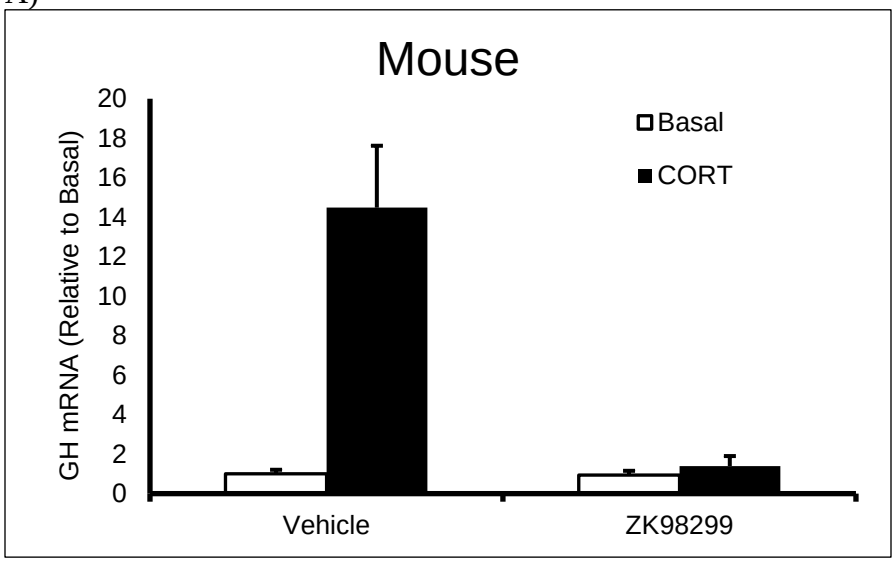
Somatotroph differentiation and GH regulation by glucocorticoids

Adrenal glucocorticoids have been shown to play an important role in somatotroph differentiation during embryogenesis (Porter, 2005). The role of adrenal glucocorticoids in somatotroph differentiation is supported by multiple studies. One

study isolated undifferentiated e12 chicken pituitary cells and showed they were induced to differentiate with the addition of e16 serum to the culture media. When serum from e12 chick embryos was added to the culture media, GH-secreting cells did not differentiate, indicating something in the e16 serum was differentiating the somatotrophs. When a glucocorticoid receptor antagonist, RU486, was additionally added with the e16 serum, the somatotrophs did not differentiate, indicating that corticosterone (CORT) was the stimulating factor in the serum (Morpurgo et al., 1997). Furthermore, unpublished data from Dr. Laura Ellestad and Dr. Tom Porter indicate glucocorticoid treatment can induce the production of *GH* mRNA and that GR is required for this response in both chickens and mice. Figure 3a presents *GH* mRNA expression from pituitary cells derived from e11 chicken pituitaries, before and after treatment with CORT (10^{-9} M final concentration), in the presence or absence of the GR antagonist ZK98299. When ZK98299 was not present in the media, CORT treatment increased *GH* mRNA expression. However, when ZK98299 was present in the media, ZK98299 blocked the response of *GH* mRNA expression to CORT treatment. Figure 3b presents data from the same experiment as figure 3a, but with pituitary cells derived from e14/15 mice embryos. When ZK98299 was not present in the media, CORT treatment increased *GH* mRNA expression. When ZK98299 was present in the media, ZK98299 blocked the response of *GH* mRNA expression to CORT treatment. This experiment suggests that CORT-induction of embryonic GH production is a mechanism common among vertebrates.



A)



B)

FIG 3. Growth hormone mRNA expression in pituitary cells derived from e11 chicken and e14/15 mice embryos A) GH mRNA expression in e11 chicken somatotrophs in response to CORT, in the absence or presence of a glucocorticoid receptor antagonist (ZK98299). B) GH mRNA expression in e14/e15 mouse somatotrophs in response to CORT, in the absence or presence of a glucocorticoid receptor antagonist. All results were normalized to GH mRNA levels in cells cultured under basal conditions (Ellestad, LE, and Porter, TE, unpublished results).

In vivo experiments showed that CORT can induce somatotroph differentiation as well. Chicken eggs (e11) were injected with either e12 or e16 serum and incubated for another three days. Reverse hemolytic plaque assays (RHPA) and immunocytochemistry (ICC) were conducted on pituitaries cells isolated from embryos of each group. The pituitary cells from eggs injected with e16 serum showed statistically significant increases in GH-containing and GH-secreting cells, indicating e16 serum is capable of differentiating somatotrophs in chicken embryos. A similar experiment was conducted, however this time e11 chicken embryos were injected with CORT or saline. Again, differentiated somatotrophs were observed, further indicating CORT induced somatotroph differentiation in chicken embryos (Bossis and Porter, 2000). Experiments utilizing dexamethasone in embryonic rats show similar results, indicating glucocorticoids can differentiate somatotrophs prematurely in mammals as well as birds. Furthermore, pharmacological inhibition of adrenal glucocorticoid synthesis in pregnant rats resulted in reduced numbers of somatotrophs on 19 dpc in the developing embryos (Nogami and Tachibana, 1993). Additionally, *in ovo* ACTH administration on e11 into chicken embryos stimulated CORT production and prematurely differentiated somatotrophs by e13 (Jenkins et al., 2007). It should be noted that somatotroph differentiation and proliferation events coincide with the rise in production of adrenal glucocorticoids during embryogenesis, summarized in figure 4 (Porter et al., 2007; Vakili and Cattini, 2012).

In summary, a large quantity of research has been conducted to investigate how glucocorticoids regulate somatotroph differentiation and GH expression in mammals and birds. Glucocorticoids can prematurely induce somatotroph

differentiation and GH expression *in vitro* and *in vivo*. This can be shown through adrenal glucocorticoids stimulated by ACTH, synthetic glucocorticoids like dexamethasone, or directly through CORT.

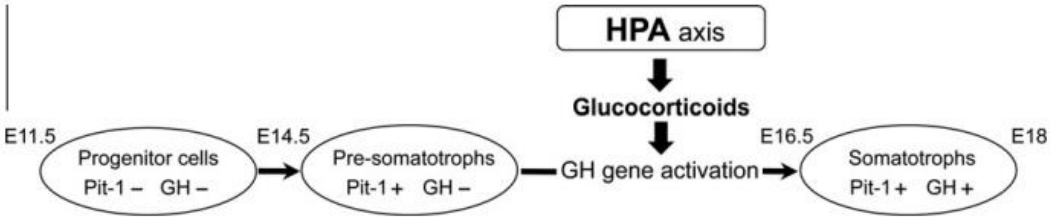


FIG 4. A schematic summary of Rathke's pouch progenitor cells committing to the somatotroph lineage and differentiating following the establishment of the hypothalamo-pituitary-adrenal (HPA) axis during mouse embryonic development (taken directly from Vakili and Cattini, 2012).

Research objectives

In summary, the pituitary gland develops during embryogenesis through a complex network of signaling factors and transcription factors that dictate cell fate, differentiation, and ultimately hormone production. Somatotrophs have been extensively studied in order to better understand the mechanisms of growth hormone expression. *In vitro* and *in vivo* experiments in rodents and chickens indicate glucocorticoids play an important role in somatotroph differentiation and growth hormone expression. Based upon this previous research, we hypothesized the glucocorticoid receptor is essential for the normal ontogeny of growth hormone during mouse embryonic development. Our objectives were to observe the effects of deleting the glucocorticoid receptor in the pituitary on growth hormone mRNA expression during mouse embryonic development by utilizing the cre-LoxP recombinase system under control of the pituitary-specific *αGSU* promoter.

Chapter 2: Pituitary-targeted knockout of glucocorticoid receptors suppresses growth hormone expression during mouse embryonic development.

Abstract

During pituitary development, establishment of the hypothalamo-pituitary-adrenal (HPA) axis and production of corticosterone (CORT), the primary glucocorticoid (GC) in rodents and chickens, has been shown to play an important role in the differentiation of somatotrophs, the endocrine cell type within the pituitary that produces growth hormone (GH), as well as *GH* mRNA expression in both avian and mammalian species. GCs bind to target cells that express the glucocorticoid receptor (GR), which in turn, acts as a transcription factor and regulates the expression of several genes. Numerous studies have been conducted on *GH* mRNA regulation and somatotroph differentiation by GCs, however few studies have specifically investigated the role of GR in the pituitary in these processes. We hypothesized that GR is essential for the normal ontogeny of GH during embryonic development. To better understand the role GR plays in somatotroph differentiation and GH regulation by GCs in a mammalian model, mouse embryos with pituitary-targeted GR knockouts were generated utilizing the cre-LoxP Recombinase system under control of the pituitary-specific *αGSU* promoter. Pituitaries, as well as brain, heart, liver, and muscle tissues, were collected on embryonic day 18/19 for RNA isolation. RT-qPCR was conducted to measure gene expression in each tissue between genotypes. *GR* mRNA and *cre* mRNA expression was measured in all tissues, and *Pit1* mRNA, *αGSU* mRNA, *GH* mRNA, prolactin (*PRL*) mRNA, thyroid-stimulating hormone beta subunit (*TSHβ*) mRNA, luteinizing hormone beta subunit (*LHβ*) mRNA, and follicle-stimulating hormone beta subunit (*FSHβ*) mRNA levels were measured in the pituitary. *Cre* mRNA expression was only found in the

pituitary, and *GR* mRNA was significantly decreased in the $GR^{(-/-)}$ embryos. *GH* mRNA was significantly decreased in $GR^{(-/-)}$ embryos, while $GR^{(+/-)}$ embryos expressed intermediate levels of *GH* mRNA in comparison to wild-type $GR^{(+/+)}$ embryos. Significant differences in expression of other pituitary hormones in $GR^{(-/-)}$ embryos were not observed, indicating that GR must not play an essential role in regulating the expression of any other pituitary hormones or cell types. Furthermore, all $GR^{(-/-)}$ embryos died at birth, or soon after. To our knowledge, this is the first study to report that homozygous GR knockout in the pituitary suppresses embryonic GH expression and results in a neonatal lethal phenotype.

Introduction

The anterior pituitary gland consists of five unique endocrine cell types, including somatotrophs, lactotrophs, gonadotrophs, thyrotrophs, and corticotrophs, which release growth hormone (GH), prolactin (PRL), luteinizing hormone (LH) and follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), and adrenocorticotrophic hormone (ACTH), respectively. The first endocrine cell type to differentiate is the corticotrophs (Treier and Rosenfeld, 1996). The production of ACTH from corticotrophs stimulates the production of glucocorticoids (GCs), specifically corticosterone (CORT) in chicken and rodents, from the adrenal glands during development. CORT travels through the circulatory system and binds to cells that express the glucocorticoid receptor (GR), which upon binding with CORT, acts as a transcription factor and regulates several genes (Kassel and Herrlich, 2007). CORT has been shown *in vitro* and *in vivo* to prematurely differentiate chicken and rat somatotrophs and induce *GH* mRNA expression (Bossis and Porter, 2000; Bossis

and Porter, 2003; Morpurgo, et al., 1997; Nogami and Tachibana, 1993; Dean and Porter, 1999; Dean et al., 1999; Porter et al., 2001; Porter and Dean, 2001; Liu et al., 2003; Liu and Porter, 2004; Fu and Porter, 2004; Bossis et al., 2004; Fu et al., 2004; Jenkins et al., 2007; Heuck et al., 2009; Heuck-Knubel et al., 2012; Jenkins et al., 2013; Ellestad et al., 2013). Based on these previous findings, we hypothesized that GR is essential for the normal ontogeny of *GH* gene expression. Previous studies have investigated the role of GCs in somatotroph differentiation and *GH* mRNA expression, however to our knowledge, none have specifically investigated the effect of GR knockout in the pituitary. This may be due to the fact that whole body knockout of GR in mice leads to neonatal death as a result of immature lungs (Tronche et al., 1998). In order to investigate the role of GR in somatotroph differentiation and *GH* mRNA expression in mice, we generated homozygous GR pituitary knockout mouse embryos utilizing the cre-LoxP recombinase system under control of the pituitary-specific *αGSU* promoter.

Materials and Methods

Mice

All studies were approved by the Institutional Animal Care and Use Committee at the University of Maryland. Wild-type C57 Black/6 mice were purchased from Charles River Laboratories or Jackson Laboratories. C57 Black/6 mice with pituitary-targeted glucocorticoid receptor (*Nr3c1)* heterozygous knockouts were created by breeding two separate transgenic mouse lines, utilizing the cre-LoxP Recombinase system. The first transgenic mouse line contained a cre cassette inserted

downstream from 4.6 kb of the mouse *αGSU* promoter (*Cga*). *αGSU* encodes for the common α-subunit of the three glycoprotein hormones that are synthesized and secreted by the anterior pituitary, including follicle-stimulating hormone (FSH), luteinizing hormone (LH), and thyroid-stimulating hormone (TSH). Each of these hormones have distinct β-subunits that form disulfide bonds with αGSU, creating the unique biological functions of each hormone. *αGSU* mRNA is easily detected in thyrotrophs and gonadotrophs of mice by e11.5, making it the earliest marker of differentiation of a hormone-producing cell in the pituitary, however it is eventually expressed throughout the entire pituitary gland (Japón et al., 1994). In this model, the *cre* recombinase gene is expressed in cells when the *αGSU* gene is expressed. The *αGSU*-cre mice were generated as previously described (Cushman et al., 2000) and were purchased from Jackson Laboratories (JAX stock #004426). The second transgenic mouse line contained LoxP sites flanking exon 2 of *Nr3c1* and were generated as previously described (Brewer et al., 2003). Exon 2 of *Nr3c1* contains the translational start site for GR. These *Nr3c1*-floxed mice were generously provided by Dr. Louis J. Muglia (Washington University School of Medicine, Saint Louis, Missouri). Figure 5 provides a schematic of the *αGSU*-cre and *Nr3c1*-floxed mice transgenes. These figures were taken directly from previously published papers (Cushman et al., 2000; Brewer et al., 2003). The *αGSU*-cre mice were bred with *Nr3c1*-floxed mice to establish offspring that were heterozygous positive for the floxed-glucocorticoid receptor (GR) and positive for *cre*, designated $GR^{(+/-)}$. Breeding pairs of $GR^{(+/-)}$ mice (ages 7-11 months) were established after genotyping confirmed all mice were $GR^{(+/-)}$ and cre-positive. Initially, females were allowed to give birth to

their litters. However, we determined that zero homozygous floxed-GR, cre-positive pups, designated as $GR^{(-/-)}$, survived. To overcome the lethality of the cre-positive/$GR^{(-/-)}$ genotype, timed pregnant females were generated. To do so, male mice were removed from breeding cages two days after their introduction to the females, and the dams were sacrificed 18 days later. As somatotroph differentiation typically occurs around embryonic day (e) 16 in mice (Japón et al., 1994), this method created an accurate fertilization timing window between these two days, confidently producing mouse pups at e18 or e19 of gestation, after the age at which somatotroph populations should be established.

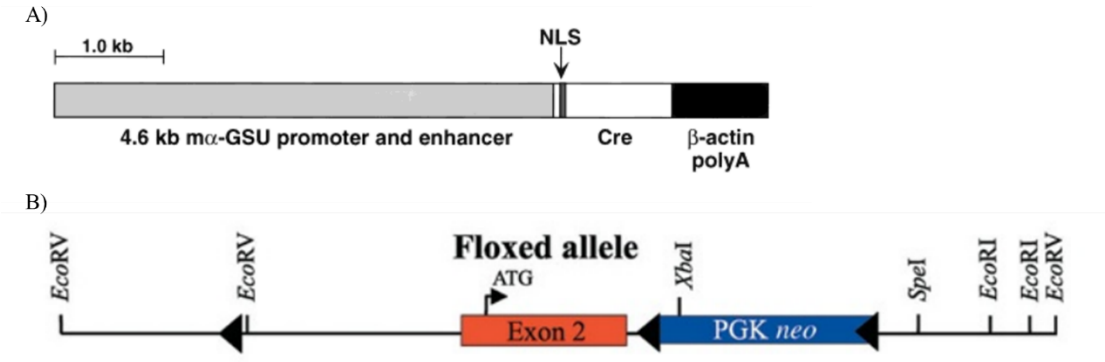


FIG 5. Schematic of *αGSU*-cre and *Nr3c1*-floxed mice transgenes. A) The *αGSU*-cre transgene was generated by placing a nuclear localization signal (NLS), the cre recombinase gene, and a *β-actin* poly-A signal downstream of the 4.6 kb mouse *αGSU* promoter and enhancer. B) The *Nr3c1*-floxed transgene was generated by inserting loxp sites flanking exon 2 of *Nr3c1* and a PGK *neo* cassette (figures taken from Cushman et al., 2000; Brewer et al., 2003).

Tissue dissection for mRNA quantification

All dissections were performed in a laminar-flow hood, and dissection tools were sterilized prior to each experiment. Pregnant mice were sacrificed at 18-19 days post-fertilization by CO_2 asphyxiation. Forceps and dissecting scissors were used to make an opening in the ventral body cavity of pregnant mice. The embryos were removed from the uterus and decapitated. The head from each embryo was placed on sterile gauze under a dissecting microscope. The cranium of each embryo was opened using sterile scissors, and the pituitary gland was removed and placed in a petri dish containing sterile MEM. Anterior pituitary glands were separated from the posterior pituitary and connecting tissue and were immediately placed into liquid nitrogen. Whole brain tissue was also collected. As the pituitary and whole brain tissue were being collected, dissecting scissors were used to make an opening in the ventral body cavity of the embryos, exposing the chest and abdominal cavities. Forceps and dissecting scissors were used to collect the liver and heart, as well as muscle tissue from the left hind-leg. Tail snips were also collected from each embryo for DNA extraction and genotyping, as described below. Forceps and dissecting scissors were washed and sterilized with 70% ethanol between each mouse embryo dissection.

DNA extraction

Genomic DNA was extracted from mouse tail snips using the QIAamp Fast DNA Tissue kit (Qiagen, Cat: 51404), according to manufacturer's instructions. Briefly, tail snips in microcentrifuge tubes were cut into smaller pieces using a clean scalpel blade. The following kit reagents were added at the following volumes to each microcentrifuge tube: 200 µl AVE, 40 µl VXL, 1 µl DX reagent, 20 µl Proteinase K,

and 4 µl RNase A (100mg/ml). Microcentrifuge tubes were shaken (270 rpm) in an incubator (37 ^{o}C) overnight to dissolve all tissue. The following day, 265 µl Buffer MVL were added to each tube and vortexed. The mixture from each microcentrifuge tube was transferred to a QIAamp mini spin column in a clean 2 ml collection tube. Collection tubes were centrifuged, and each spin column was placed into a new collection tube (All centrifuge steps were performed for 30 seconds at max speed unless stated otherwise). Buffer AW1 (500 µl) was added to each spin column, and all collection tubes were centrifuged. Spin columns were placed into new collection tubes. Buffer AW2 (500 µl) was added to each spin column, and all collection tubes were centrifuged. All spin columns were transferred to new collection tubes and centrifuged for 2 minutes at maximum speed to remove any residual buffer from the spin columns. After centrifugation, all spin columns were placed into sterile 1.5 ml microcentrifuge tubes. DNA elution buffer (50 µl) was carefully pipetted directly onto the filter of each spin column. After one minute of incubation at room temperature, all microcentrifuge tubes were centrifuged for one minute at max speed to collect the purified DNA.

PCR genotyping

PCR reactions (20 µl) were conducted to genotype each mouse. Working solutions containing the forward and reverse primers (10 µM) were made for mouse *gr* (WT), flanking loxp sequences (FLOX), and *cre* (CRE). Mouse *gh* (GH) primers were used as an internal positive control for each PCR reaction. Samples previously determined to be negative for either *gr*, loxp sequences, or *cre*, were used as negative controls for specific plates to provide confidence that no contamination was present in

the reactions. Optimized PCR reaction recipes were the same for the FLOX primers and the WT primers. For individual sample reactions, 1 µl of template DNA (30-50ng/µl) was added to a well containing 0.3 µl of GH primers, 1 µl of WT or FLOX primers, and 17.7 µl of GoTaq (Promega). When samples were genotyped for CRE, 1 µl of template DNA (30-50 ng/µl) was added to a well containing 0.3 µl CRE primers, 1 µl GH primers, and 17.7 µl GoTaq. Table 1 provides sequences for all primers used to genotype the mice. A thermocycler was set with the following cycling program: A 5 min step at 95 ºC, followed by 35 cycles comprising of 95 ºC for 45 secs, 57 ºC for 30 sec, and 72 ºC for 1 min. After 35 cycles, a final 72 ºC extension step occurred for 7 minutes before being lowered to 4ºC.

Gene	Forward Primer (5’ to 3')	Reverse Primer (5' to 3')
GH	CCCTCATCCTAGTGAACAAACA	AGTTGGAACGCACTCACATTA
GR (WT)	GGCATGTTAGAAACTGGAAAGGA	CAGTTCTTAACCCTCTCATTGAAAGGT
GR (FLOX)	GGCATGTTAGAAACTGGAAAGGA	CAATAGCAGGCAACAACTTCGT
CRE	CGATGCTTTTAAACCTGTGAGAGTT	CACGTAACAGACGTTTTCAGATACCT

Table 1. Primers used for PCR to genotype mouse DNA.

Gel electrophoresis

Once the PCR reaction was completed, 20 µl of 1X loading buffer (Biorad) was added to each well. A clean, rubber seal was placed on individual PCR plates to allow the plates to be vortexed. After reaction wells were mixed, the entire plate was centrifuged and 8 µl from each well was loaded into a 1% agarose gel for electrophoresis. Duplicate reactions for each sample and primer combination were run in adjacent wells.

1% agarose gels

Agarose (2.5 grams, Genesee, Cat:20-102) was dissolved in a flask containing 250 ml of TE buffer and 7.5 µl of ethidium bromide (10 mg/ml, Biorad, Cat:20-102). Once the gel solidified, PCR plates were loaded into wells with a DNA ladder at the end of each lane. Gels were run at 130 V for 60-90 minutes.

Total RNA extraction

Total RNA was extracted using a RNeasy Mini Kit (Qiagen, Cat: 74106) according to manufacturer's instructions. Mouse tissue samples were removed from a -80 ºC freezer and placed into liquid nitrogen. The homogenizer (Scilogex) was cleaned before the first sample, in between each sample, and after the last sample was homogenized. Briefly, a 500ml beaker was placed underneath the homogenizer to collect all liquids used in cleaning. With the homogenizer turned on, 0.2% SDS was sprayed on the homogenizer tip, followed by autoclaved distilled water, RNase zap (Invitrogen, Carlsbad, CA), and autoclaved distilled water again. A sterile tissue wipe was used to dry off the tip of the homogenizer. Individual samples were removed

from liquid nitrogen using hemostats. Buffer RLT (600 µl) was added to each sample. The homogenizer tip was used to gently push sample tissue toward the bottom of the cryotube to submerge it in the buffer. Samples were then homogenized for approximately 30 seconds, gently mixing the cryotube up and down. On-column DNase digestion was used to reduce levels of contaminating DNA. Samples were eluted in 60 µl of RNase-free water and quantified using the Quant-it RiboGreen assay kit (Invitrogen).

Analysis of mRNA levels by reverse transcription-quantitative PCR (RT-qPCR)

Two-step RT-qPCR was performed to quantify mRNA levels in each embryonic tissue (e18/19) across genotypes. Every reverse transcription reaction (20 µl) consisted of 1 µl of 50 µM oligo-dT primer, 1 µl of 10mM dNTPs, 4 µl of 5X first strand buffer, 1 µl of 0.1 M DTT, 1 µl of an RNase inhibitor (10U/µl), 1 µl of SuperScript III (200U/µl) and 1 µg of T-RNA, except for the mouse pituitary samples due to low T-RNA recovery (50-200 ng). Negative controls for genomic DNA contamination were created by pooling RNA from each tissue and processing them in the same way as the other samples, but without SuperScript III (No RT control). All reactions were diluted with 180µl of RNase-free water, except the mouse pituitary reactions, which were diluted with 20 µl of RNase-free water. Each PCR reaction (15µl) consisted of 0.6 µl forward primer, 0.6 µl reverse primer, 5.3 µl of autoclaved distilled water, 7.5 µl of 2X QuantiTect SYBR Green PCR master mix (Qiagen), and 1 µl of cDNA.

Annealing temperatures for each primer varied slightly, however, the remaining thermal cycling parameters were held consistent and are as follows: 1 cycle

of 95 ºC for 15 minutes, followed by 40 cycles of 95 ºC for 10 sec, 54 or 55 ºC for 30 sec, and 75 ºC for 30 sec. Finally, a melt curve cycle held at 65 ºC for 5 sec and then increased to 95 ºC in 0.5 ºC increments was performed. Primer sequences of each gene used in RT-qPCR are listed in Table 2. Levels of *β-actin* (*ACTB*) mRNA were quantified in every sample. Statistical analysis indicated levels of mRNA differed by tissue ($P < 0.05$) but did not differ by genotype within any individual tissue. These results confirm *β-actin* was a suitable gene for normalization of individual mRNA levels within each tissue. After normalization, all data were analyzed using the $2^{-\Delta\Delta Ct}$ equation, where Ct equates to the cycle number when the amount of amplified cDNA product reached a fixed threshold of fluorescence for each sample. Data for cre and GR mRNA expression are presented across all tissues by genotype. Data for mRNA levels for pituitary hormones are individually presented in the pituitary samples by genotype.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
Mouse		
β-actin	GATTACTGCTCTGGCTCCTAGCAC	GACAGTGAGGCCAGGATGGA
GH	TTCTAATGCTGTGCTCCGAGC	AATGGAATAGCGCTGTCCCTC
TSHβ	GGAGAGAGTGTGCCTACTGCCT	CCTGAGAGAGTGCATATTTGGGA
PRL	AGAAGCCCCCGAATACATCC	TCCCATTTCCTTTGGCTTCA
POMC	CCATAGATGTGTGGAGCTGGTG	TCCAGCGAGAGGTCGAGTTT
LHβ	CCCAGTCTGCATCACCTTCA	TAGGTGCACACTGGCTGAGG
FSHβ	ACCAGCTTTCCCTCACATGC	CAGGTGTGTTTGTAGGCAAGCTAA
α-GSU	TTCCAAAGAATATTACCTCGGAGG	GCTACAGTGGCACTCCGTATGAT
Pit1	CAAACGAAAGGAAGAGGAAACG	AGCCATCCGCATGATCTCC
GR	GAGGACAACCTGACTTCCTTG	AACTCACATCTGGTCTCATTCC
CRE	GCTGGAGTTTCAATACCGGAGA	CATTGCCCCTGTTTCACTATCC

Table 2. Primers used for reverse-transcription, quantitative PCR.

Statistical Analyses

Cre and *GR* mRNA expression data from e18/19 mouse embryos are reported as mean ± SEM for each genotype for each tissue. Data were analyzed by one-way analysis of variance within tissue using the PROC ANOVA procedure in SAS (Statistical Analysis System; Cary, NC). Tukey's post-hoc analysis was conducted to determine statistical significance between genotypes for each tissue. *αGSU*, *β-actin*, *Pit1*, Growth hormone (*GH*), Thyroid-stimulating hormone beta subunit (*TSHβ*), Follicle-stimulating hormone beta subunit (*FSHβ*), Luteinizing hormone beta subunit (*LHβ*), Prolactin (*PRL*), and Proopiomelanocortin (*POMC*) mRNA expression data from e18/19 mouse embryos are reported as the means ± SEM for each genotype from pituitary tissue. Data were analyzed by one-way analysis of variance using the PROC ANOVA procedure in SAS. Tukey's post-hoc analysis was conducted to determine statistical significance between genotypes for each gene.

Results

Relative cre mRNA expression across tissues

Figure 6 presents relative *cre* mRNA expression, normalized to *β-actin*, by tissue and genotype. A two-way ANOVA was conducted to compare *cre* mRNA expression across all tissues and genotypes. *Cre* mRNA expression was only detected in the pituitary, and it was significantly greater than all other tissues ($P = 0.0001$). A one-way ANOVA was conducted on the pituitary data, followed by a Tukey's test for post-hoc analysis. Significant differences between genotypes for each tissue are shown using letters. *Cre* mRNA expression was not statistically different among any

genotypes within the pituitary tissue (P = 0.06). *Cre* mRNA expression was largest (0.028) in the wild-type ($GR^{(+/+)}$) embryos (n=3). *Cre* mRNA expression of the floxed ($GR^{(-/-)}$) embryos (n=3) was 0.013. *Cre* mRNA expression of heterozygous ($GR^{(+/-)}$) embryos (n=5) was 0.015. However, these apparent differences were not statistically different. These results indicate that cre expression was restricted to the pituitary gland and did not differ among genotypes on e18/19.

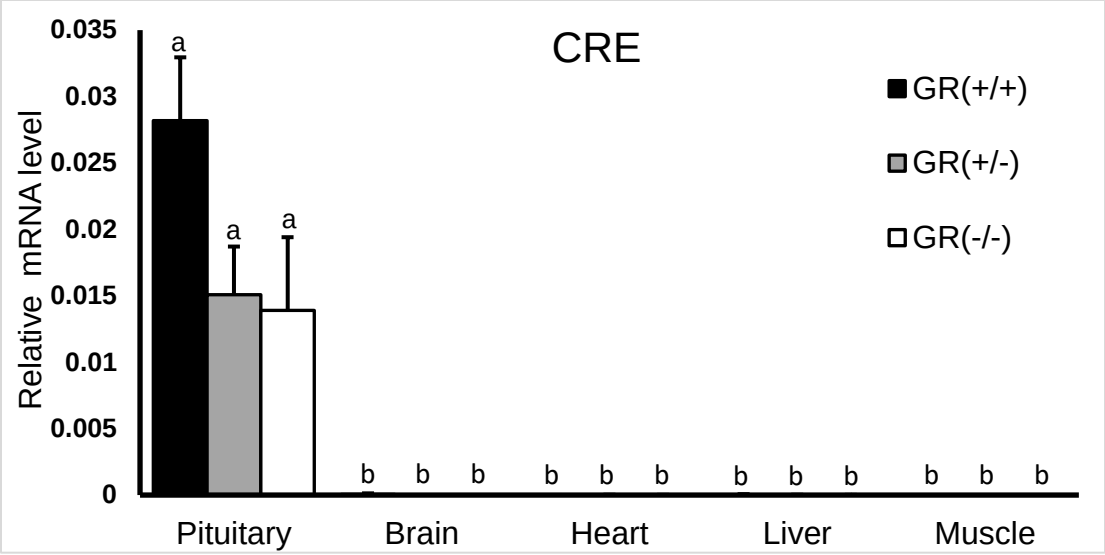


FIG 6. Relative *cre* mRNA expression across tissues by genotype in e18/19 mice.

Relative GR mRNA expression across tissues

Figure 7 presents relative *GR* mRNA expression, normalized to wild-type *β-actin*, by tissue and genotype. One-way ANOVAs were conducted for each tissue, followed by Tukey's tests for post-hoc analysis. Significant differences between genotypes for each tissue are shown using different letters. There were no significant differences in *GR* mRNA expression between any genotype for the brain (P = 0.11) and the muscle (P = 0.14). *GR* mRNA expression in the heart of $GR^{(-/-)}$ mouse embryos (n=3) was significantly less (P = 0.002) compared to $GR^{(+/-)}$ mice (n=5) and $GR^{(+/+)}$ mice (n=3). In the liver, *GR* mRNA expression in $GR^{(+/+)}$ mouse embryos was significantly greater (P = 0.004) compared to $GR^{(+/-)}$ and $GR^{(-/-)}$ mice. Importantly, *GR* mRNA expression for each genotype was significantly different from each other in the pituitary (P = 0.0001), with levels for $GR^{(+/+)}$ embryos being the greatest, $GR^{(+/-)}$ embryos being intermediate, and $GR^{(-/-)}$ embryos being the lowest. These results indicate that *GR* mRNA was suppressed in the pituitary, heart, and liver of $GR^{(-/-)}$ mice.

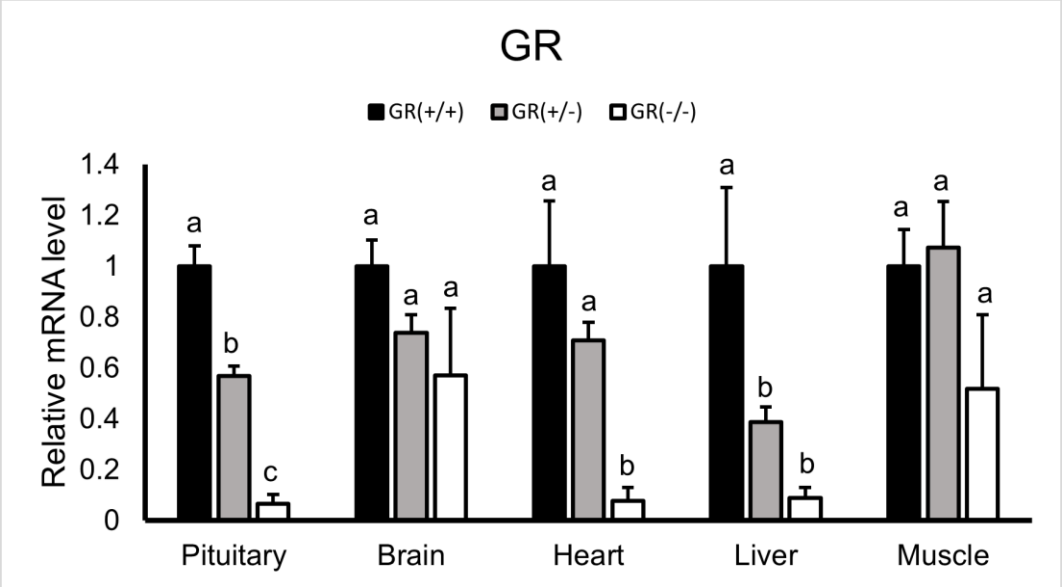


FIG 7. Relative *GR* exon2 mRNA expression across tissues by genotype in e18/19 mice normalized to WT *β-actin* within each tissue.

Relative pituitary hormone mRNA expression

Figure 8 presents relative mRNA expression of multiple pituitary-related genes by genotype, including growth hormone (*GH*), prolactin (*PRL*), pituitary-specific transcription factor 1 (*Pit1*), proopiomelanocortin (*POMC*), alpha glycoprotein subunit (*αGSU*), thyroid-stimulating hormone beta (*TSHβ*), luteinizing hormone beta (*LHβ*), and follicle-stimulating hormone beta (*FSHβ*). One-way ANOVAs were conducted for each gene, followed by Tukey's tests for post-hoc analysis. Significant differences between genotypes for each tissue are shown using different letters. Expression of *GH* mRNA was significantly reduced (P = 0.01) in $GR^{(-/-)}$ mice (n=3) compared to wild-type (n=3) mice. Interestingly, levels of *GH* mRNA in heterozygous mice (n=5) were intermediate, not significantly different from either wild-type or floxed mice. *PRL* (P = 0.53), *Pit1* (P = 0.65), *POMC* (P = 0.12), *αGSU* (P = 0.38), *TSHβ* (P = 0.46), *LHβ* (P = 0.17), and *FSHβ* (P = 0.31) were all not significantly different among genotypes. Although levels of mRNA for *POMC*, *LHβ*, and *FSHβ* tended to be different between genotypes, these differences were not significant with the number of litters we analyzed. These results indicate that of the hormone-related genes analyzed only *GH* mRNA levels were significantly reduced in the pituitary glands of $GR^{(-/-)}$ mice.

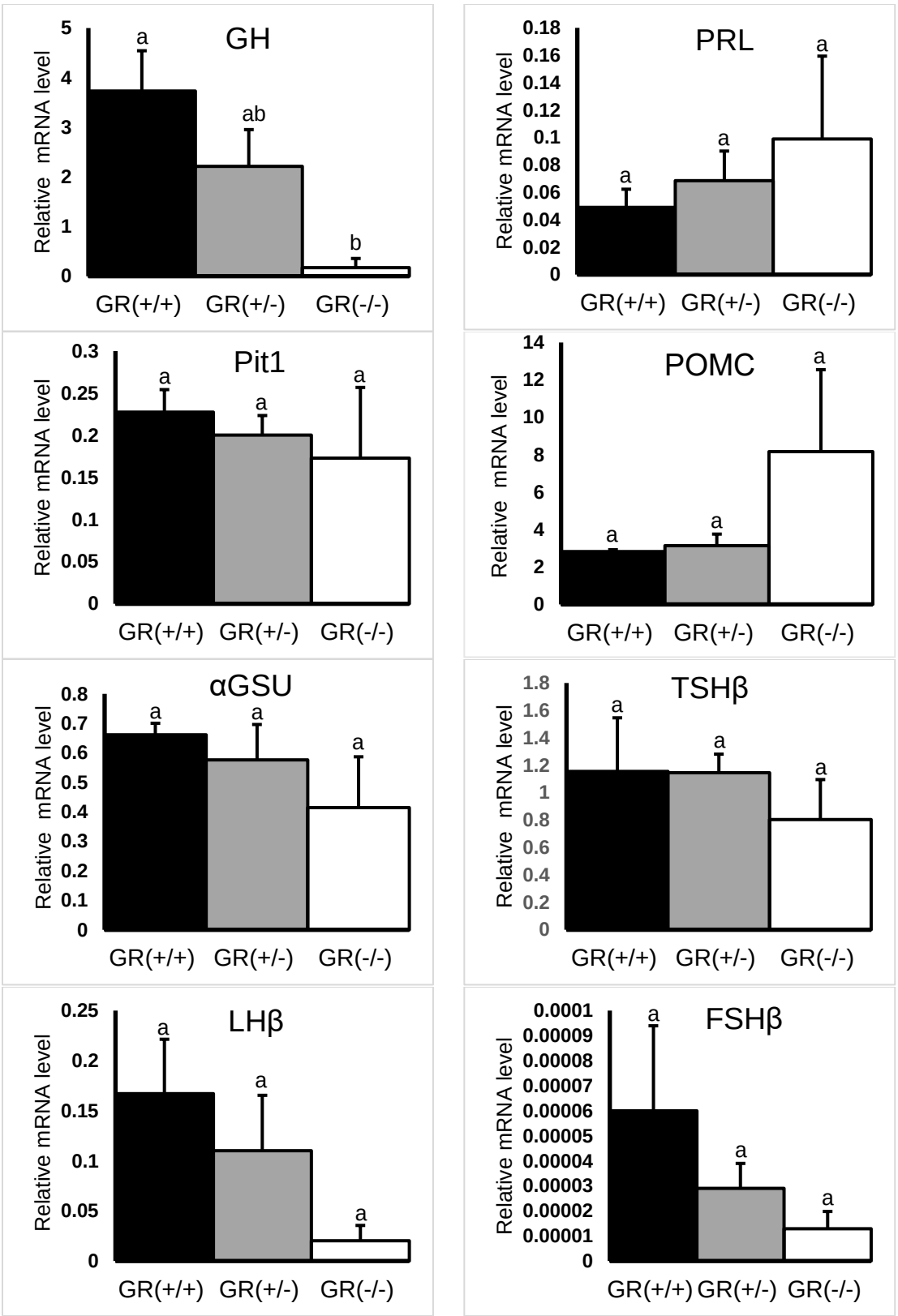


FIG 8. Relative mRNA expression of pituitary genes in the pituitary of e18/19 mice.

Pituitary-targeted knockout of GR is a neonatal lethal phenotype in mice

Table 3 presents genotypes of pups and e18/19 embryos from cre-positive, heterozygous $GR^{(+/-)}$ breeding pairs. Table 3a presents genotyping data of pups from $GR^{(+/-)}$ breeding pairs from three separate paternal family lines. DNA samples from pups were collected between 10-28 days of age. Pup genotyping data (n=14) were analyzed as cre positive or negative for each genotype ($GR^{(+/+)}$, $GR^{(+/-)}$, $GR^{(-/-)}$). Zero pups were genotyped as cre negative-$GR^{(+/-)}$, cre negative-$GR^{(-/-)}$, or cre positive-$GR^{(-/-)}$. 36% of the pups (n=5) were cre negative-$GR^{(+/+)}$. 57% of the pups (n=8) were cre positive-$GR^{(+/-)}$. Only 7% of the pups (n=1) were cre positive-$GR^{(+/+)}$. When only cre positive pups (n=9) were analyzed by genotype, 0% of the pups were $GR^{(-/-)}$, 11% of the pups were $GR^{(+/+)}$ (n=1), and 89% of the pups were $GR^{(+/-)}$ (n=8). These results indicate that no cre positive-$GR^{(-/-)}$ mice were present after birth.

Table 3b presents genotyping data from e18/19 embryos from cre-positive $GR^{(+/-)}$ breeding pairs from three paternal family lines. Embryo genotyping data (n=18) were analyzed as cre positive or negative for each genotype ($GR^{(+/+)}$, $GR^{(+/-)}$, $GR^{(-/-)}$). Similar to data from Table 3a, zero embryos were found to be cre negative-$GR^{(+/-)}$ or cre negative-$GR^{(-/-)}$. Among all embryos, 22% of the embryos (n=4) were cre negative-$GR^{(+/+)}$, 33% of the embryos (n=6) were cre positive-$GR^{(+/-)}$, and 28% of the embryos (n=5) were found to be cre positive-$GR^{(+/+)}$. Interestingly, 17% of the embryos (n=3) were found to be cre positive-$GR^{(-/-)}$. When only the cre positive embryos (n=14) were analyzed by genotype, 43% were $GR^{(+/-)}$ (n=6), 36% were $GR^{(+/+)}$ (n=5), and 22% were $GR^{(-/-)}$ (n=3). Thus, $GR^{(-/-)}$ embryos survived to e19 but died around the time of birth.

A)

Paternal Family	Total # of pups on day of birth	GR(+/-) ; CRE (-)	GR(+/+) ; CRE (-)	GR(-/-) ; CRE (-)	GR(+/-) ; CRE (+)	GR(+/+) ; CRE (+)	GR(-/-) ; CRE (+)
E	6	0	2	0	3	1	0
		0%	33%	0%	50%	17%	0%
C	4	0	2	0	2	0	0
		0%	50%	0%	50%	0%	0%
G	4	0	1	0	3	0	0
		0%	25%	0%	75%	0%	0%
	Average %	0%	36%	0%	57%	7%	0%
	% among CRE+ pups				89%	11%	0%

B)

# of litters	Total # of embryos on e18/19	GR(+/-) ; CRE (-)	GR(+/+) ; CRE (-)	GR(-/-) ; CRE (-)	GR(+/-) ; CRE (+)	GR(+/+) ; CRE (+)	GR(-/-) ; CRE (+)
3	18	0	4	0	6	5	3
	Average %	0%	22%	0%	33%	28%	17%
	% among CRE+ pups				43%	36%	22%

Table 3. Table of genotypes by percent from $GR^{(+/-)}$ breeding pairs A) Genotypes by percent from pups born from separate paternal family lines. B) Genotypes by percent from embryos collected on e18/19.

Discussion

The regulation of growth hormone (*GH*) mRNA expression by glucocorticoids (GCs) has been extensively studied in the past, however contradicting results have led to some discrepancies as to whether glucocorticoids negatively or positively regulate *GH* mRNA expression. Traditionally, GCs are thought to negatively regulate *GH* mRNA expression (Allen, 1996). For example, individuals diagnosed with Cushing's disease, a state of continuous cortisol overproduction, display low levels of *GH* mRNA production, along with reduced growth, and other metabolic disorders (Tritos, 2021). However, a growing body of evidence argues that GCs are important for somatotroph differentiation and GH production (Fu and Porter, 2004; Bossis and Porter, 2003; Porter et al., 2001; Bossis and Porter, 2000; Ellestad et al., 2009). The different effects of GCs on GH expression are most likely explained either by differences between species, *in vivo* versus *in vitro* data, developmental stage or physiological state of an animal, the concentration of GCs, and long-term versus short-term effects (Vakili and Cattini, 2012).

Since chicken embryos develop in incubated eggs rather than *in utero*, stages of development can be accurately timed, and embryos can be easily manipulated. Embryos can be incubated for various lengths of time after manipulation. The same cannot be said for mice or rats, which is most likely why the majority of previous literature investigating CORT effects of GH regulation and somatotroph differentiation has been conducted *in vitro*, using chickens as a model.

Despite numerous studies on glucocorticoids prematurely inducing differentiation of somatotrophs, the mechanism is not thoroughly understood. Studies

exploring this mechanism in chicken embryonic pituitary cells indicate that CORT leads to increased *GH* transcriptional activity, rather than increased mRNA stability. Ras and ERK1/2 signaling have been shown to be involved in CORT induction of *GH* during embryogenesis as well (Ellestad et al., 2015). In the unpublished cell culture work from Dr. Laura Ellestad and Dr. Tom Porter, CORT was added as a treatment, and GR was demonstrated to be necessary for CORT to elicit a *GH* response using a GR antagonist. Thus, substantial evidence has implicated CORT and GR in the regulation of GH expression and somatotroph differentiation. However, a requirement for GR has not been evaluated in vivo using a knockout animal model. Investigating the effects of a pituitary-targeted $GR^{(-/-)}$ knock-out chicken model on *GH* mRNA expression would be impractical because of the difficulties in creating transgenic chickens. Therefore, we generated a pituitary-targeted GR knock-out ($GR^{(-/-)}$) mouse model to investigate the role of GR in embryonic *GH* expression in a mammalian model.

Mice positive for αGSU-cre and heterozygous ($GR^{(+/-)}$) for the floxed GR allele were bred, and mouse embryos (e18/19) were dissected out of dams to isolate brain, pituitary, heart, liver, and muscle tissue. Tail snips were collected for DNA isolation to genotype the embryos. Reverse-transcription reactions were conducted on RNA isolated from all tissues, allowing for RT-qPCR analysis of mRNA levels for each tissue among genotypes. In the pituitary, both *GR* and *GH* mRNA expression was significantly decreased in the cre-positive, homozygous GR knock-out ($GR^{(-/-)}$) mouse embryos. We speculate that both copies of the *GR* gene (*Nr3c1*) must be actively transcribed in wild-type ($GR^{(+/+)}$) mice pituitaries because cre-positive $GR^{(+/-}$

$^{)}$ mice, which possess only one copy of *Nr3c1* due to cre expression knocking out the other copy, displayed intermediate levels of *GH* mRNA. This is supported by our findings that *GR* mRNA levels in the pituitary of $GR^{(+/-)}$ mice were significantly different from *GR* mRNA levels in the pituitary of both $GR^{(+/+)}$ and $GR^{(-/-)}$ mice. These results demonstrate an essential role for GR in the pituitary for *GH* mRNA expression and somatotroph differentiation.

We also analyzed several other pituitary-related genes to observe if knocking-out GR had any additional effects on their expression. The POU homeodomain protein PIT1, a pituitary-specific transcription factor, is expressed in mice by e15.5 (Simmons et al., 1990). *Pit1* is believed to be essential for proper differentiation of somatotrophs, thyrotrophs, and lactotrophs since they are not observed in *Pit1* mutant mice. Additionally, *GH*, thyroid-stimulation hormone (*TSH*), and prolactin (*PRL*) mRNA is not detected in *Pit1* mutant mice either (Li et al., 1990). When we analyzed *Pit1* mRNA expression, we found no significant differences between genotypes. These results provide evidence that GR knockout does not alter *Pit1* mRNA expression. Therefore, the reduction of *GH* mRNA levels observed in $GR^{(-/-)}$ mice is likely due to a lack of GR and not due to effects on PIT1.

When we analyzed the expression of *αGSU, FSHβ, LHβ*, and *TSHβ*, we found no significant differences between genotypes in *αGSU* or *TSHβ* mRNA expression. Interestingly, *FSHβ and LHβ* mRNA seemed to be reduced in $GR^{(-/-)}$ embryos, however with our sample size, this difference in gene expression was not significantly different between genotypes. These results indicate that GR does not regulate the expression of *αGSU, FSHβ, LHβ,* or *TSHβ* mRNA in the mouse during embryonic

development. We also analyzed the expression of proopiomelanocortin (POMC) and prolactin (PRL) in the pituitary. We found no significant differences in gene expression for any of these genes, across all genotypes. Noteworthy, *POMC* mRNA expression appeared to be upregulated in $GR^{(-/-)}$ mice, although this was not statistically significant. *POMC* mRNA expression may have been upregulated if the negative feedback loop between glucocorticoids and the hypothalamic-pituitary-adrenal (HPA) axis was interrupted, however, GR would have had to be knocked out in the paraventricular nucleus of the hypothalamus, since the paraventricular nucleus is the major location of negative feedback from glucocorticoids (Laryea et al., 2013). Expression of *GR* mRNA in brain tissue did not show any significant differences between any genotypes, therefore it is unlikely GR was knocked out in the paraventricular nucleus of the hypothalamus. Our findings indicate that pituitary-targeted knockout of GR suppressed mRNA levels in the pituitary for only *GH* but not the other pituitary hormone genes.

Based on these findings in our transgenic model, we conclude that GR expression in the pituitary is required during embryogenesis for normal *GH* ontogeny in mice. Additionally, other pituitary-related genes analyzed in this study, including *Pit1*, *PRL*, *POMC*, *αGSU*, *TSHβ*, *FSHβ* and *LHβ*, were not significantly different between genotypes. Thus, the effect of pituitary-targeted knockout of GR was specific to *GH*, suppressing normal *GH* expression during embryonic development. To the best of our knowledge, this is the first *in vivo* investigation to demonstrate that GR expression in the pituitary is required during embryogenesis for *GH* mRNA expression and somatotroph differentiation in mouse embryos.

From the onset of this study, we discovered that the *GR* gene was not following Mendelian genetics in our mouse pups. Genotyping data of pups from cre-positive $GR^{(+/-)}$ breeding pairs from three separate paternal family lines were analyzed to determine the number of cre positive pups that belonged to each genotype. Our results showed nine out of fourteen total pups expressed cre. Of these nine pups, eight were genotyped as $GR^{(+/-)}$ (89%), one was $GR^{(+/+)}$ (11%), and zero were $GR^{(-/-)}$ (0%) (Table 3). During embryogenesis, however, the heredity pattern of the *GR* gene indicated that Mendelian genetics were being followed. When genotyping data of e18/19 embryos from cre-positive $GR^{(+/-)}$ breeding pairs from three separate litters were analyzed to determine the number of cre-positive embryos that belonged to each genotype, our results showed fourteen out of eighteen total embryos expressed cre. Of these fourteen embryos, six were $GR^{(+/-)}$ (43%), five were $GR^{(+/+)}$ (36%), and three were $GR^{(-/-)}$ (22%). If a larger sample size was available, we speculate the heredity pattern of the glucocorticoid receptor gene during embryogenesis would distinctly follow Mendelian genetics.

To a certain extent, the observed difference in heredity of the *GR* gene between the embryos and the pups is due to the *αGSU* promoter driving *cre* expression. In cells that expressed *αGSU*, the cre recombinase enzyme would have been translated. If embryos inherited one copy of GR gene that was flanked by two *loxP* sites (floxed), the cre enzyme would catalyze recombination between the *loxP* sites and remove the floxed region of the *GR* gene in that cell. This would lead to the generation of heterozygous $GR^{(+/-)}$ mice. If embryos inherited two copies of the floxed GR gene, the embryos would be homozygous-negative $GR^{(-/-)}$.

Be that as it may, Mendelian genetics were not followed in the pups that survived birth because at some point between embryonic day 18/19 and birth, or soon after, all of the $GR^{(-/-)}$ embryos died. It should be noted that dams consume dying or weak pups, therefore collecting DNA from $GR^{(-/-)}$ embryos to verify their genotypes was logistically difficult. These results indicate that homozygous knockout of GR in the pituitary is a neo-natal lethal phenotype, however the explanation for embryo death is unknown. Previous studies utilizing the *αGSU* promoter in mice to drive *cre* expression have reported *cre* expression on e9.5 in tissues outside of the pituitary, including in muscle and cardiac tissue, with little or no expression in gonads, adrenal glands, kidneys, brain, or ventromedial hypothalamus (Cushman et al., 2000). While the only tissue we detected *cre* expression in was the pituitary on e18/19, we did see *GR* mRNA significantly suppressed in the heart of $GR^{(-/-)}$ embryos, as well as significantly reduced levels in the liver of $GR^{(+/-)}$ and $GR^{(-/-)}$ embryos compared to $GR^{(+/+)}$ embryos. While *GR* mRNA expression in the liver was the lowest in the $GR^{(-/-)}$ embryos, it was not significantly different from the $GR^{(+/-)}$ embryos. A previous study demonstrated knocking out *GR* in cardiomyocytes led to cardiac hypertrophy and death, however, these mice appeared normal at birth, and did not show symptoms of heart failure until around 5 months of age (Oakley et al., 2013). A different study that conducted *in-situ* hybridization in adult mouse tissues, using β-galactosidase as a reporter gene for *αGSU* expression, found no significant expression in the liver, however, the β-galactosidase level was not considered significant because it was not greater than 2-fold over control levels (Kendall et al., 1994). It should be noted that if *αGSU* is expressed at any period in a cell, cre recombinase will be synthesized and

excise the floxed region of the *GR* gene. Therefore, it is theoretically possible *cre* was expressed in cardiac and liver tissue early in development through the *αGSU* promoter, and the floxed region of the *GR* gene was removed from the genome of these cells, but we did not detect *cre* on e18/19 because *αGSU* is no longer expressed in the tissue at that point. While *αGSU* is traditionally considered to be a pituitary-specific gene, these results, as well as previous reports, indicate the ontogeny and tissue distribution of *αGSU* expression may not be completely understood.

GR has been shown to be necessary for lung development in mice as well (Laryea et al., 2013). Since we did not collect lung tissue from our embryo samples, we can only speculate if improper lung development contributed to neo-natal lethality. Furthermore, it would have been interesting to analyze circulating levels of CORT from each embryo and compare them across genotypes to determine if the HPA axis was dysfunctional within the $GR^{(-/-)}$ embryos. It is clear, however, that *GH* and *GR* mRNA expression were significantly decreased in $GR^{(-/-)}$ embryos and that all $GR^{(-/-)}$ pups died at birth or shortly thereafter. Appendix A provides genotyping data from thirteen generations of mice that survived to adulthood and one generation of embryos collected on e18/19. Zero mice from the thirteen generations that survived to adulthood genotyped as WT-negative, FLOX-positive, and CRE-positive. These data support our claim that the WT-negative, FLOX-positive, and CRE-positive genotype ($GR^{(-/-)}$) is a neonatal lethal phenotype. In summary, the *GR* gene followed Mendelian genetics during embryogenesis but did not follow mendelian genetics in the pups that survived after birth because $GR^{(-/-)}$ embryos died at or soon after birth. While the precise mechanisms that led to the observed neo-natal lethal phenotype in $GR^{(-/-)}$

embryos is unknown, we argue that neonatal lethality is a direct consequence from the homozygous knockout of the glucocorticoid receptor in the pituitary or another tissue.

In conclusion, our findings indicate that pituitary-targeted knockout of GR suppresses the normal ontogeny of *GH* mRNA expression during mouse development. This finding, in combination with the substantial evidence that CORT can induce premature expression of GH during embryonic development, indicates an essential role for CORT and GR in normal somatotroph differentiation.

Chapter 3: Effects of growth factors on Rathke's pouch development in chicken embryos.

Introduction

The pituitary gland is derived from two separate ectoderm layers, neural ectoderm and oral ectoderm. Oral ectoderm evaginates cranially to form Rathke's pouch, the precursor structure to the anterior pituitary. Neural ectoderm evaginates caudally soon after Rathke's pouch forms, contributing to the future hypothalamus and posterior pituitary. The physical contact and cell signaling that occurs between these two layers during Rathke's pouch formation is essential for proper pituitary development. Retinoic acid has been shown to be essential for correct pituitary morphology and plays a role in *Prop1* expression (Yoshida et al., 2018). Sonic hedgehog signaling acts as a morphogen and plays an important role in Rathke's pouch positioning and formation. Studies in mice have shown that a lack of sonic hedgehog signaling within the neural ectoderm results in two Rathke's pouch formations (Trowe et al., 2013). Based on these previous studies, we hypothesized that microinjections of retinoic acid and sonic hedgehog agonists may increase Rathke's pouch tissue area and length, while retinoic acid receptor and sonic hedgehog inhibitors may reduce tissue area and length. Our objectives were to evaluate the direct effects of growth factors on Rathke's pouch morphology.

Materials and Methods

Chicken eggs

Fertilized Ross broiler chicken eggs were obtained from Perdue Farms (Salisbury, MD) and set in a humidified incubator (37.5 ºC, 40-50% humidity) on

their side for 36-40 hours. Eggs were further incubated (same conditions) for 24 hours after microinjections.

Pulling injection needles

Microinjection needles were pulled with a P-97 Flaming/Brown micropipette puller (Sutter Instrument Company, Novato, CA). The needles used for microinjections were thin-wall borosilicate capillary glass with microfilament, 4 inches long, 1 mm outside diameter, 0.75 mm inside diameter (A-M Systems, Sequim, WA, USA).

Windowing embryos

After eggs incubated for 36-40 hours, 3M packaging tape, approximately 2.5" x 2.5", was placed over the eggs, with the corners of the tape making a shape resembling a diamond to prevent the shell and shell debris from falling into the egg when the shell is cut and removed. A 10cc syringe with an 18-guage needle was used to pierce the shell at the blunt end of each egg at a 45-degree angle, to carefully remove 5-6 ml of albumin from the bottom of the egg, as to not disturb the orientation of the egg yolk and developing embryo. An oval-shaped cut was made in the eggshell with dissection scissors from the hole in the egg created by the needle when albumin was removed. Albumin was slowly added back into the egg to raise the yolk to the surface of the egg. India ink was injected underneath the yolk membrane using a 1cc syringe with a 22-guage needle attached to visualize the embryo. Before injecting the India ink, the needle was bent 45 degrees using the cap to provide the proper angle needed for injections. From here, the forebrain of each embryo was microinjected

with experimental treatments. Figure 9 provides an example of before and after microinjection of embryonic day 1.5 chicken embryos. After microinjections, 5-6mls of albumin was removed to lower the embryo. A plastic bulb pipette was used to place 3-4 drops of sterilized PBS containing antibiotics into the egg to prevent bacteria growth. Parafilm was used to cover the hole in the eggshell and eggs were placed back into incubators for 24 hours. After 24 hours, the embryos were collected and processed through a paraffin wax embedding protocol, described below.

Treatments

Retinoic acid (Sigma, 50 mg, Cat: 302-79-4, RA) and AGN194310 (Sigma, 5 mg, Cat: 229961-45-9), a retinoic acid receptor antagonist (RARA) were diluted (10^{-6} M final concentration) in PBS for microinjection to test their effects on Rathke's pouch formation. Jervine (Caymen Chemical, 1 mg, Cat: 469-59-0), an inhibitor of the Sonic Hedgehog pathway, and SAG (Caymen Chemical, 1 mg, Cat: 912545-86-9), an agonist of Smoothened, were also diluted (10^{-6} M final concentration) to further test their effects on Rathke's pouch formation.

Paraffin Embedding, Histology, and Sectioning

Embryos were removed from incubators 24 hours after microinjections. Albumin (5-6 ml) was added back to the eggs to raise the yolk to the surface of the eggshell. Filter paper was cut into rings and carefully placed around the embryos. Dissection scissors were used to carefully cut around the embryo. Tweezers were used to transfer embryos out of the egg. Once embryos were successfully transferred, they were processed through the following series of washes for at least 1 hour to

prepare them for paraffin embedding: 10% formaldehyde, 70% ethanol, 95% ethanol, 100% ethanol, and xylene. Following all wash steps, embryos were embedded in the sagittal plane in paraffin wax. Paraffin wax blocks were sectioned (10 µm) and stained with hematoxylin and eosin (Histoserv, Germantown, MD).

Image Capture and Analysis

Midsagittal sections were identified by observing when Rathke's pouch appeared and disappeared throughout consecutive slides. Images of midsagittal sections were captured utilizing a Leica DMI6000B microscope with a DFC420C camera and analyzed with ImageJ software (NIH). Figure 10 presents examples of midsagittal sections of Rathke's pouch of embryonic day 2.5 chicken embryos. Length was measured by drawing a straight line from the bottom right of Rathke's pouch directly to the tip of Rathke's pouch. Tissue area was measured by tracing the outline of the entire pouch. Total area was measured by tracing the outline of the entire pouch including a straight line across the bottom of Rathke's pouch to include the space between Rathke's pouch.

Statistical Analysis

Data for each graph are reported as mean ± SEM from each treatment group. Data were analyzed by one-way analysis of variance using the PROC ANOVA procedure in SAS (Statistical Analysis System; Cary, NC). Tukey's post-hoc analysis was conducted to determine statistical significance between treatment groups.

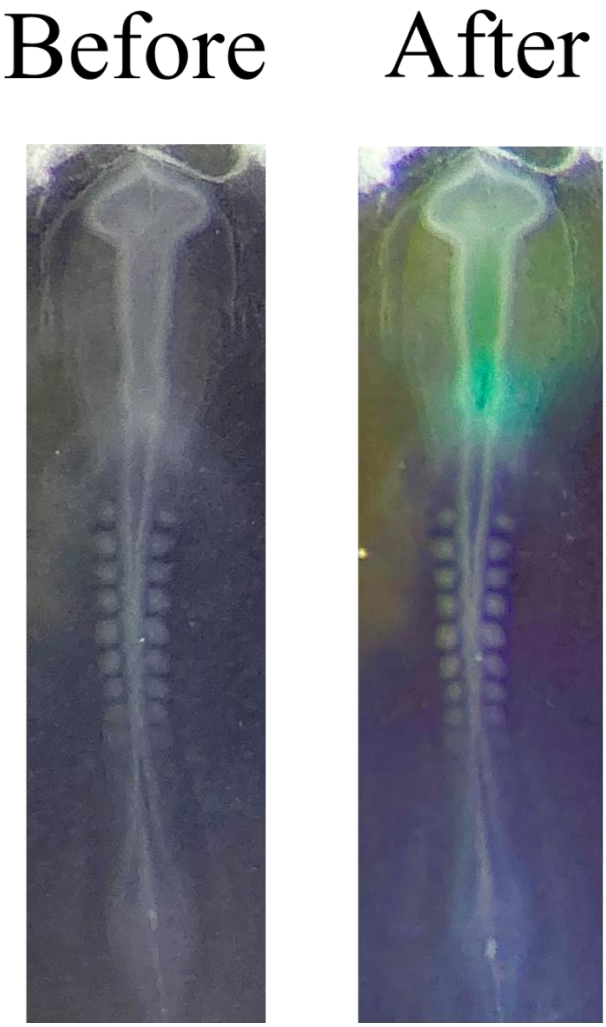


FIG 9. Before and after microinjection of embryonic day 1.5 chicken embryos.

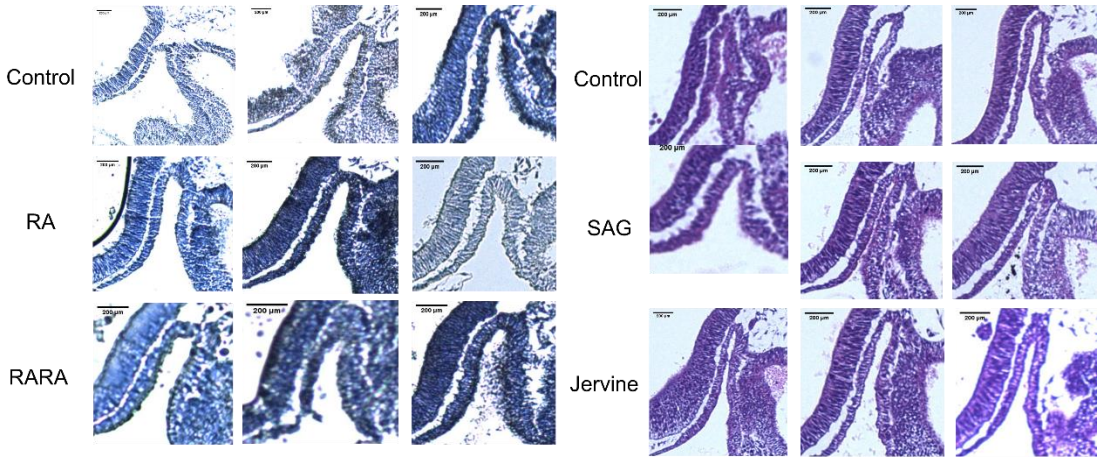


FIG 10. Midsagittal sections of Rathke's pouch of embryonic day 2.5 chicken embryos.

Results

Retinoic acid and retinoic acid receptor antagonists

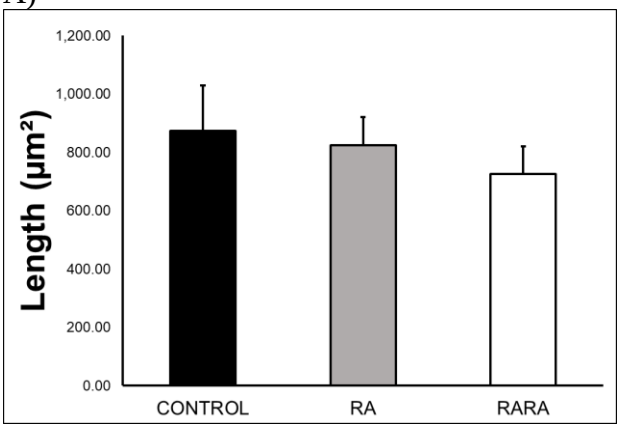
RA and RARA were microinjected into the neural tube of e1.5 chicken embryos to test their effect on RP formation. Figure 11 shows average length, tissue area, and total area between control, retinoic acid (RA), and retinoic acid receptor antagonist (RARA) treated groups. Figure 11a presents the average length of Rathke's pouch for control, RA, and RARA treated groups. Figure 11b presents average tissue area of Rathke's pouch for control, RA, and RARA treated groups. Figure 11c presents average total area of Rathke's pouch for control, RA, and RARA treated groups. RA and RARA-treated embryos showed reduced RP length, tissue area, and total area compared to controls, however no significant differences were observed between any treatment group.

Smoothened agonist (SAG) and Sonic hedgehog antagonist (Jervine)

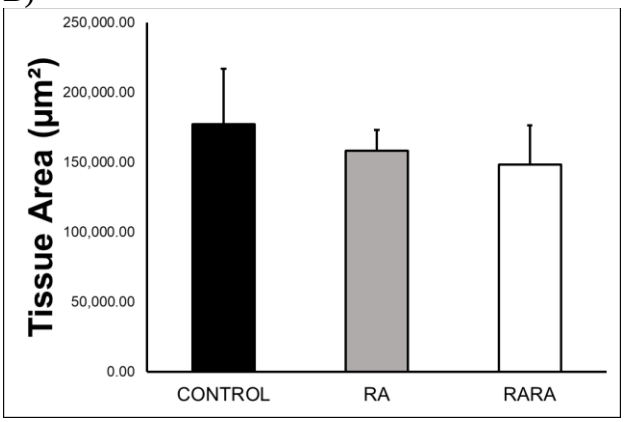
SAG and Jervine were microinjected into the neural tube of e1.5 chicken embryos to test their effect on RP formation. Figure 12 shows the average length, tissue area, and total area between control, a sonic hedgehog antagonist (Jervine), and a sonic hedgehog agonist (SAG). Figure 12a presents average length of Rathke's pouch for control, jervine, and SAG treated groups. Figure 12b presents average tissue area of Rathke's pouch for control, jervine, and SAG treated groups. Figure 12c presents average total area of Rathke's pouch for control, jervine, and SAG treated groups. Compared to controls, SAG treatment reduced RP length, tissue area, and

total area by 47%, 51%, and 65% respectively ($P < 0.05$; n=3). Jervine-treated embryos were not statistically different from controls.

A)



B)



C)

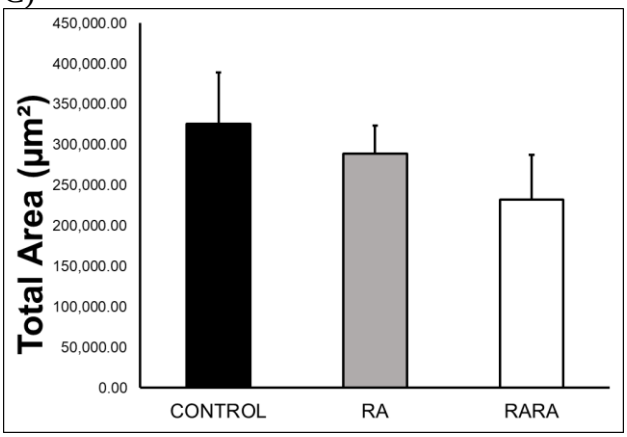
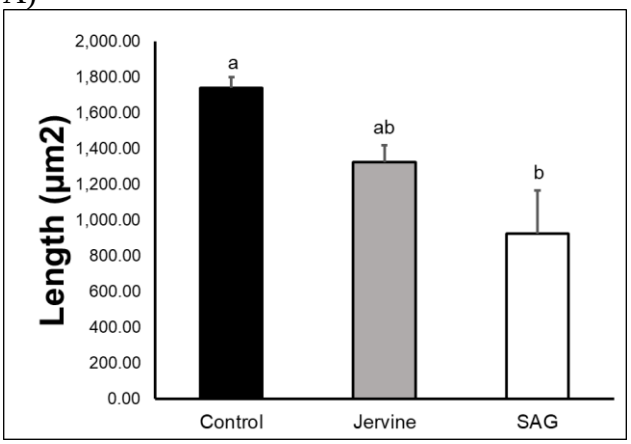
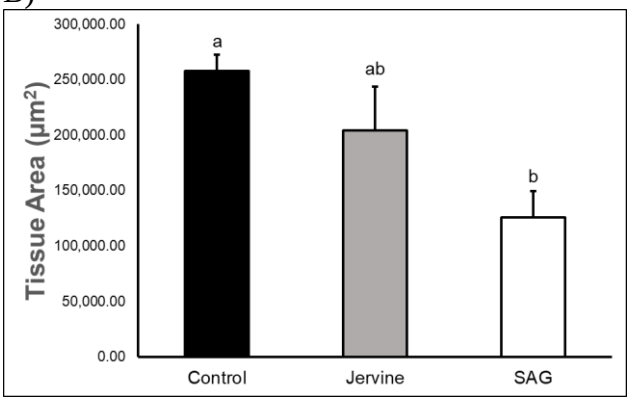


FIG 11. Average length, tissue area, and total area between control, retinoic acid (RA), and retinoic acid receptor antagonist (RARA) treated groups. A) Average length of Rathke's pouch for control, retinoic acid (RA), and retinoic acid receptor antagonist (RARA) treated groups. B) Average tissue area of Rathke's pouch for control, retinoic acid (RA), and retinoic acid receptor antagonist (RARA) treated groups. C). Average total area of Rathke's pouch for control, retinoic acid (RA), and retinoic acid receptor antagonist (RARA) treated groups.

A)



B)



C)

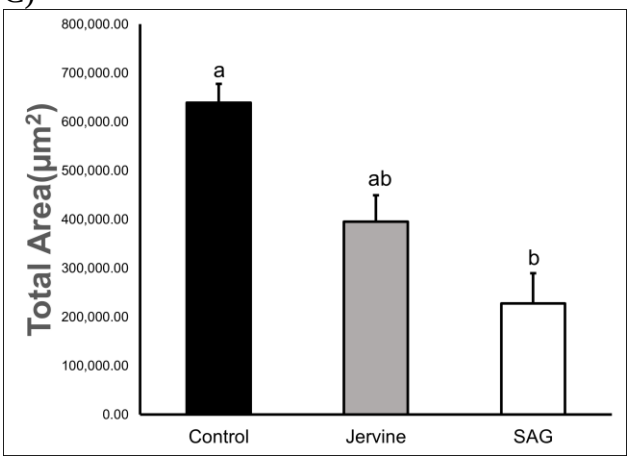


FIG 12. Average length, tissue area, and total area between control, a sonic hedgehog antagonist (Jervine), and a sonic hedgehog agonist (SAG). A) Average length of Rathke's pouch for control, Jervine, and smoothened agonist (SAG) treated groups. B) Average tissue area of Rathke's pouch for control, Jervine, and smoothened agonist (SAG) treated groups. C) Average total area of Rathke's pouch for control, Jervine, and smoothened agonist (SAG) treated groups.

Discussion

Retinoic acid, and its receptor antagonist, were chosen as treatment groups because retinoic acid has been shown to play a role in *Prop1* expression (Yoshida et al., 2018). Control groups were simply microinjected with PBS. While RA and RARA-treated groups trended to decrease in length, tissue area, and total area, these results were not statistically significant from the control groups.

SAG and Jervine were also microinjected to investigate the role of the sonic hedgehog signaling pathway on RP formation. Previous experiments utilizing SAG have shown to induce embryogenic mouse stem cells into Rathke's pouch-like cells *in vitro* (Suga et al., 2011). Therefore, we hypothesized that SAG treatment may result in increased RP measurements or resemble control treatment embryos. Interestingly, SAG-treated groups showed significant decreases in RP length, tissue area, and total area compared to control, the opposite of what was hypothesized.

Jervine has been used in clinical studies to investigate its role in reducing cancer cell proliferation by inhibiting the sonic hedgehog pathway (Carballo et al., 2018; Zhao et al., 2021). Based on these previous studies, we hypothesized that Jervine-treated embryos may result in reduced RP measurements. While we did see reductions in RP measurements, these differences were not significant compared to controls.

Chapter 4: Future directions

There are several different approaches that could conducted in the future that would provide additional data and improve the overall quality of the previously described research experiments. First, in regard to the pituitary-targeted glucocorticoid receptor knockout mice experiment, the establishment of additional breeding pairs and paternal lines would be beneficial in order to increase the sample size of each genotype. During our experiment, we generated three different paternal lines and utilized timed pregnant females. Embryos were collected if a female mouse was verified pregnant, which was done by visually inspecting and physically palpating the abdomen a few days prior to a planned experiment. However, breeding pairs had to be reestablished multiple times due to females not becoming pregnant, which required a significant amount of time. In the future, other methods could be used to verify pregnancy in order to conduct multiple experiments in a more time efficient manner, such as setting up cameras to monitor individual breeding cages for mating behavior. The establishment of additional paternal lines would also increase the chances of female mice becoming pregnant and increase sample sizes within individual litters.

Furthermore, the neonatal lethal phenotype observed in the homozygous GR knockout mice could not be explained. While knocking out GR in the lungs in mice is known to be a neonatal lethal phenotype, lung tissue was not collected at the time of these experiments because *α-GSU* is not thought to be expressed in lung tissue. Since lung tissue was not collected in the previous experiments, we cannot rule out the possibility of GR being knocked out in the lung. Future studies should consider

collecting lung tissue in order to determine if GR mRNA is present within the tissue. This would provide more information related to the neonatal lethal phenotype, as well as provide a better understanding of *α-GSU* mRNA expression outside of the pituitary during mouse embryonic development.

Additionally, it would be interesting to further investigate how the HPA axis is affected in the homozygous GR knockout mice. In the previous experiment, *POMC* mRNA expression trended to be greater in the homozygous knockout mice, however it was not significantly greater from the other genotypes. Therefore, it is unknown if the HPA axis was disrupted in the knockout mice or if our sample size was too small to measure significance. Future studies should collect blood at the time of dissection and measure circulating plasma CORT levels within each sample and compare across each genotype. This would add data that could be used to further determine in the HPA axis was disrupted. If CORT levels are significantly greater in the homozygous GR knockouts, this could be explained by a disruption in the negative feedback loop of CORT on *POMC* mRNA expression in corticotrophs within the pituitary, resulting in *POMC* mRNA and CORT levels to be greater in the homozygous GR knockout mice compared to the other genotypes.

In addition to analyzing mRNA levels of pituitary hormones within the pituitary, conducting RT-qPCR on genes for the receptors for hypothalamic releasing hormones, such as the growth hormone releasing hormone receptor, may have provided more relevant information to this project.

Other experimental approaches could be conducted in the future to further investigate hormone production within the pituitary in these knockout mice. Pituitary

histology sections could be created, and antibody staining could be utilized to visualize *GH* mRNA or protein expression within the pituitary and compare between genotypes. This approach could also be utilized at varying days of development and be used to analyze other tissues for GR to see if it was truly knocked out. It would also be beneficial to further investigate cre expression at earlier time points in development and measure cre expression in the tissues where GR was knocked down.

Future experiments regarding Rathke's pouch development in chicken embryos should target other signaling pathways, such as WNT/β-catenin, Notch, and BMP signaling. This could easily by conducted by utilizing different treatments at different concentrations. Furthermore, different incubation lengths following microinjections could be tested.

While these treatments may in fact affect Rathke's pouch development, it is possible the microinjection method itself is not practical. Embryos were examined one hour after microinjection and the treatment solution appeared to be diffused out from the neural tube, indicating the treatments may not be maintained within the neural tube and that this method may not be feasible. In an attempt to overcome these practical challenges, nanobeads could be used in a similar fashion in previous studies (Szabo-Rogers et al., 2008; Chambers et al., 2000). This would potentially allow sources of concentrated growth factors or inhibitors to slowly diffuse while being maintained within the neural tube.

Appendices

Generation 1

Mouse	Wt	Flox	Cre	Mother-Family	Father-Family
PD 404	X	X	X	X	X
PD 385	X	X	X	X	X

Generation 2

Mouse	Wt	Flox	Cre	Mother-Family	Father-Family
WT1	+	-	-	CR	CR
PD 449	-	+	+	PD 404	PD 385

Generation 3

Mouse	Wt	Flox	Cre	Mother-Family	Father-Family
PD 721	+	+	+	WT1	PD 449
WT 14	+	+	+	JAX	JAX

Generation 4

Mouse	Wt	Flox	Cre	Mother-Family	Father-Family
PD 806	+	+	+	PD 721	WT 14
PA 385	X	X	X	CR	CR

Generation 5

Mouse	Wt	Flox	Cre	Mother-Family	Father-Family
PD 867	+	+	+	PA 385	PD 806
PA 405	X	X	X	CR	CR

Generation 6

Mouse	Wt	Flox	Cre	Mother-Family	Father-Family
PD 937	+	+	+	PD 867	PA 405
PA 391	X	X	X	CR	CR

Generation 7

Mouse	Wt	Flox	Cre	Mother-Family	Father-Family
PD1003	+	+	+	PA 391	PD 937
PA 408	X	X	X	X	X

Generation 8

Mouse	Wt	Flox	Cre	Mother-Family	Father-Family
PD 1204	+	+	+	PA 408	PD 1003
PA 454	+	-	-	X	X
PA 440	+	-	-	X	X

Generation 9

Mouse	Wt	Flox	Cre	Mother-Family	Father-Family
PD 1362	+	+	+	PA 454	PD 1204
PD 1302	+	+	+	PA 440	PD 1204

Generation 10

Mouse	Wt	Flox	Cre	Mother-Family	Father-Family
PD 1458	+	+	+	PD 1302	PD 1362/G
PD 1240	+	+	+	PA 418/423	PD 1208
PD 1076	+	+	+	PD 985	PA 408
PA 480	+	-	-	X	X
PA 481	+	-	-	X	X
PA 415	+	-	-	X	X
1771 HAMZA	+	-	-	X	X

Generation 11

Mouse	Wt	Flox	Cre	Mother-Family	Father-Family
PD 1511	+	+	+	PA 480	PD 1458/G
PD 1525	+	+	+	PA 481	PD 1458/G
PD 1243	+	+	+	PD 1240	1771 HAMZA
PD 1218	+	+	+	PA 415	PD 1076/C

Generation 12

Mouse	Wt	Flox	Cre	Mother-Family	Father-Family
PD 1579	+	+	+	PD 1525/G	PD 1511/G
2150 HAMZA	+	-	-	X	X
PD 1481	+	+	+	PA 460	PD 1243/A
2148 HAMZA	+	-	-	X	X
2064 HAMZA	+	-	-	X	X
PD 1490	+	+	+	PA 471	PD 1218/C
2087 HAMZA	+	-	-	X	X

Generation 13

Mouse	Wt	Flox	Cre	Mother-Family	Father-Family
PD 1660	+	+	+	2150	PD 1579/G
PD 1654	+	+	+	2148	PD 1481/A
PD 1609	+	+	+	2064	PD 1579/G
PD 1652	+	+	+	2148	PD 1481/A
PD 1621	+	+	+	2087	PD 1490/C

Generation 14

Mouse	Wt	Flox	Cre	Mother-Family	Father-Family
PD 1654 E4	+	+	+	PD 1654/A	PD 1660/G
PD 1654 E6	+	+	+	PD 1654/A	PD 1660/G
PD 1654 E7	+	–	+	PD 1654/A	PD 1660/G
PD 1654 E8	–	+	+	PD 1654/A	PD 1660/G
PD 1621 E2	–	+	+	PD 1621/C	PD 1609/G
PD 1621 E3	+	–	+	PD 1621/C	PD 1609/G
PD 1621 E4	+	+	+	PD 1621/C	PD 1609/G
PD 1621 E5	+	+	+	PD 1621/C	PD 1609/G
PD 1621 E6	+	–	+	PD 1621/C	PD 1609/G
PD 1652 E1	+	+	+	PD 1652/A	PD 1609/G
PD 1652 E3	–	+	+	PD 1652/A	PD 1609/G

Appendix A. Genotyping data from 14 generations of breeding pairs and their offspring.

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