THE ROLE OF NOTCH SIGNALING IN THE REGULATION OF PROLIFERATION AND CELL DIFFERENTIATION DURING EMBRYONIC AND POSTNATAL PITUITARY DEVELOPMENT

BY

LEAH B. GOLDBERG

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Molecular and Integrative Physiology in the Graduate College of the University of Illinois at Urbana-Champaign, 2014

Urbana, Illinois

Doctoral Committee:

Associate Professor Lori Raetzman, Chair
Professor Ann Nardulli
Professor Phil Newmark
Assistant Professor Eric Bolton
ABSTRACT

The pituitary develops in two distinct waves in mice, one occurring during embryogenesis and a second taking place during early postnatal development. During both periods, progenitor cells proliferate and subsequently differentiate into hormone-producing cells. Maintaining the proper number of progenitors and producing the appropriate number and type of endocrine cells is essential to pituitary development and function. Although signals necessary for initial pituitary commitment and those required for pituitary hormone synthesis have been well characterized, pathways that regulate progenitor proliferation and cell fate remain unclear. Previous studies have implicated the Notch signaling pathway in these processes, both in the pituitary and in other developing tissues. In order to more specifically define the role of Notch signaling during different stages of pituitary development, we utilized pituitary-specific gain- and loss-of-function mouse models, in combination with chemical Notch inhibition.

Notch signaling is necessary for progenitor maintenance in many systems. Additionally, loss of the canonical Notch target Hes1 in combination with loss of the pituitary specific Notch target, Prop1, results in premature differentiation of corticotropes. Based on these observations, we hypothesized that Notch signaling is sufficient to inhibit the differentiation of corticotropes and maintain these cells in a progenitor state. To test this hypothesis, we generated mice with persistent expression of activated Notch in POMC-expressing cells. We show that constitutive Notch expression prevents the differentiation of corticotropes and melanotropes, which also contain POMC. These cells instead maintain
progenitor markers. In addition, mRNA levels of transcription factors necessary for *Pomc* expression are downregulated in this model. Cells that aberrantly maintain progenitor markers are lost during early postnatal development, resulting in dysfunction of the hypothalamic-pituitary-adrenal axis. These findings confirm our hypothesis that Notch signaling is sufficient to prevent corticotrope differentiation.

Previous data have suggested that Notch signaling may be able to transcriptionally activate *Prop1*, which is necessary for *Pit1* transcription, which is necessary for GH, TSH and Prl synthesis. These findings, in combination with the observation that Notch signaling is sufficient to inhibit differentiation of POMC-expressing cells, led us to hypothesize that Notch signaling may regulate the balance between POMC lineage cells and Prop1/Pit1 lineage cells. Additionally, we hypothesized that alterations in Notch signaling may lead to changes in proliferation and progenitor maintenance, both well characterized roles of Notch. To address these hypotheses, we examined a mouse model with loss of *Notch2* prior to pituitary induction. In this model, we observe a decrease in Pit1-positive cell number and an increase in corticotrope cell number, suggesting Notch regulates the choice between these pituitary lineages. Additionally, we observe defects in proliferation and progenitor maintenance, but not until after birth, indicating *Notch2* is dispensable for these functions during embryonic development. Because of this observation, we examined the role of Notch signaling during postnatal development using a chemical inhibitor of Notch, which was specifically administered during postnatal. We observe an increase in *Pomc*
mRNA, a decrease in Pit1 mRNA and a decrease in the number of proliferating cells after Notch inhibition. These findings demonstrate that Notch signaling is an important regulator of cell fate and proliferation in the postnatal pituitary. In order to identify targets that may be necessary for these functions, microarray analysis was performed comparing Notch2 ablated mice to controls. Among the target mRNAs that were altered, many have been implicated in cell proliferation and a subset are involved in corticotrope cell signaling. These targets will be areas of future focus in the Raetzman laboratory. Taken together, these studies have defined a role for Notch signaling in the control of cellular proliferation, progenitor maintenance and specification during embryonic and postnatal pituitary development.
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Lori Raetzman, for her unending support and encouragement. I am truly grateful for her unwavering commitment to her graduate students and her fantastic mentorship. Her passion for science is truly evident and it was a great honor to learn from her.

This work could not have been accomplished without the assistance of many wonderful people. I am thankful that many of my years in lab were spent with my colleague, Paven Aujla. I appreciate her scientific insight and her encouragement throughout the years. I also thank Pamela Monahan and Tyler Moran who guided me through my first year in the Raetzman Lab and helped to teach me many of the techniques utilized in my studies. Ashley Himes contributed greatly to this work and I enjoyed our many interactions during our time in the lab together. I am grateful to have had the opportunity to work with Katherine Brannick, whose positive attitude was contagious and helped to make the lab a fun place to be. I would also like to thank Matt Biehl, Kirsten Eckstrum and Whitney Edwards, for our many scientific conversations and for making lab a positive environment. I had the pleasure of working with two outstanding undergraduate students, Agata Parfieniuk and Dan Getz, who were both dedicated to this project and helped with many of the studies.

I appreciate the scientific insight and guidance from the members of my committee, Drs. Ann Nardulli, Eric Bolton and Phil Newmark.

I am grateful for the willingness of many to share their reagents and equipment. Without their generosity, these studies would not have been possible.
I am also thankful for the funding and support provided to me through the Cell and Molecular Biology Training Grant.

Finally, I am grateful to family and friends who have supported me on this journey. I thank my parents, who instilled in me a love of science from a young age and who always encouraged me to follow my dreams. I am also indebted to my husband, Matt, for his unwavering love, commitment and encouragement.
# TABLE OF CONTENTS

**Chapter 1: Introduction** .................................................................................................................. 1

References ........................................................................................................................................... 12
Figures ................................................................................................................................................ 18

**Chapter 2: Persistent expression of activated Notch inhibits corticotrope and melanotrope differentiation and results in dysfunction of the HPA axis** ...................... 20

Abstract ............................................................................................................................................. 20
Introduction ......................................................................................................................................... 21
Materials and Methods ..................................................................................................................... 25
Results ................................................................................................................................................ 30
Discussion .......................................................................................................................................... 38
References ........................................................................................................................................... 43
Figures ................................................................................................................................................ 50

**Chapter 3: Notch signaling in postnatal pituitary expansion: proliferation, progenitors and cell specification** ..................................................................................................................... 58

Abstract ............................................................................................................................................. 58
Introduction ......................................................................................................................................... 59
Materials and Methods ..................................................................................................................... 62
Results ................................................................................................................................................ 67
Discussion .......................................................................................................................................... 79
References ........................................................................................................................................... 85
Tables and Figures ............................................................................................................................ 92

**Chapter 4: Notch signaling regulates proliferation and cell fate determination genes in the pituitary** ........................................................................................................................................ 102

Abstract ............................................................................................................................................. 102
Introduction ......................................................................................................................................... 103
Materials and Methods ..................................................................................................................... 105
Results ................................................................................................................................................ 109
Discussion .......................................................................................................................................... 114
References ........................................................................................................................................... 118
Tables and Figures ............................................................................................................................ 122

**Chapter 5: Conclusions/Discussion** ............................................................................................. 130

References ........................................................................................................................................... 138
Figure .................................................................................................................................................. 142
CHAPTER 1: Introduction

The pituitary gland, often referred to as the master gland, is an important regulator of many physiological processes including growth, metabolism, fertility and the body’s response to stress. The pituitary regulates these processes through the release of hormones, which act on target endocrine organs to regulate the subsequent release of their hormones. Proper pituitary development is necessary for pituitary function and the function of its target organs. Inappropriate development of the gland can lead to multiple disorders including Cushing’s syndrome, gigantism and combined pituitary hormone deficiency. Additionally, pituitary tumors are present in roughly 12% of the population upon autopsy, while the presence of clinically apparent tumors is 1:1100 (Daly, et al. 2006; Fernandez, et al. 2010; Raappana, et al. 2010), indicating the pituitary is highly susceptible to defects in proper control of cell proliferation. Because of the prevalence of pituitary disease, developing both during embryogenesis and during postnatal gland homeostasis, it is of great importance to understand the mechanisms and signaling pathways that underlie pituitary cell proliferation and differentiation.

In mice, the pituitary is composed of three lobes: the posterior lobe (PL), the anterior lobe (AL), and the intermediate lobe (IL). The posterior lobe, which is of neural origin, contains pituicytes and axon terminals of magnocellular neurons. The AL contains five hormone-producing cell types: thyrotropes, somatotropes, lactotropes, corticotropes and gonadotropes, which produce the hormones thyroid stimulating hormone (TSH), growth hormone (GH), prolactin (PRL),
adrenocorticotropic hormone (ACTH) and gonadotropins (LH and FSH), respectively. The IL contains a sixth hormone-producing cell type, the melanotrope. Melanotropes secrete melanocyte-stimulating hormone (αMSH). αMSH and ACTH are both cleavage products of the prohormone Proopiomelanocortin (POMC) (Zhu, et al. 2007).

The AL and IL are derived from cells located in hypophyseal placode, which is found at the midline of the anterior neural ridge. The hypophyseal placode is of ectodermal origin and expresses the transcription factor Foxg1. At the earliest stages, the hypophyseal placode is in direct contact with neural tissue that will develop into the PL and hypothalamus. This ectoderm invaginates at embryonic day (e) 9.0, forming the primordial pituitary. At e11.0, the developing pituitary pinches off from the underlying oral ectoderm to form the structure known as Rathke’s Pouch (RP). Throughout development, this tissue remains in close contact with the neural tissue that makes up the infundibulum (Rizzoti. 2010). Signals from this tissue are necessary for pituitary development. In fact, genetic ablation of this region in Nkx2.1 knockout mice, results in failure of the pituitary to form. This phenotype is likely due, at least in part, to the loss of FGF morphogens that are normally secreted by cells in the infundibulum (Kimura, et al. 1996) (Figure 1.1).

RP contains all of the progenitor cells necessary to form the AL and IL (Zhu, et al. 2007; Rizzoti. 2010). These progenitor/stem cells express the factors SOX2, and later SOX9 (Fauquier, et al. 2008). Using in vivo lineage tracing experiments, it has been shown that SOX2 and SOX9-expressing cells can give
rise to all of the different cell types of the pituitary. They can carry out this function during embryogenesis and, at a lower rate, in adult mice (Andoniadou, et al. 2013; Rizzoti, et al. 2013). Several factors have been identified that are necessary for the proper proliferation of these progenitors. These signals include both extrinsic factors emanating from the surrounding ventral diencephalon and oral ectoderm, such as members of the FGF, BMP, SHH and Wnt families, and intrinsic pituitary signals, (Ericson, et al. 1998; Treier, et al. 1998) (Figure 1.1). Despite the identification of these signaling pathways, nothing is known about how a progenitor cell integrates and translates the signals it receives to alter proliferative behavior.

At e11.5, progenitor cells begin to undergo cell cycle exit, migrate ventrally towards the developing AL and begin to differentiate into hormone producing cells (Zhu, et al. 2007; Bilodeau, et al. 2009). Many current models of pituitary development propose hormone cells are specified in an ordered fashion, with each cell type exiting the cell cycle during a discrete period of time. Furthermore, it is widely accepted that cells exiting the cell cycle at the same time migrate to the same location in the pituitary. This process is thought to be coordinated by opposing gradients of morphogens in the infundibulum and ventral diencephalon and the underlying oral ectoderm (Treier, et al. 1998; Ericson, et al. 1998). However, recent evidence shows that all of the AL hormone cell types exit the cell cycle at the same time, with the majority of cells doing so between e11.5 and e13.5. The IL melanotropes exit the cell cycle shortly thereafter. Additionally, there was found to be no correlation between the timing of cell specification and
location in the gland (Davis, et al. 2011). Based on these observations, it remains unclear how cells in the same location in the pituitary, and therefore exposed to the same chemical gradients, are specified to become different cell types.

Although all hormone cells exit the cell cycle at roughly the same time, corticotropes are the first cell type to express markers of terminal differentiation, with Pomc mRNA detectable beginning at e11.5 (Japón, et al. 1994). Transcription of Pomc is dependent on several transcription factors including TPIT (TBX19), a T-box transcription factor. TPIT cooperates with PITX1, a homeodomain transcription factor, to regulate transcription of the Pomc gene. It does so by binding directly to the Pomc promoter (Lamolet, et al. 2001; Liu, et al. 2001). Mice deficient for Tpit fail to express POMC, although corticotrope cell commitment is unaffected (Pulichino, et al. 2003a; Pulichino, et al. 2003b).

Similarly, human TBX19 mutations can result in isolated ACTH deficiency, which can be attributed to lack of POMC in the pituitary (Pulichino, et al. 2003a; Vallette-Kasic, et al. 2004). Additionally, NeuroD1, a basic helix loop helix transcription factor, can also bind synergistically with PITX1 to the Pomc promoter and activate its transcription. Mice null for Neurod1 show delayed expression of ACTH but eventually show normal hormone production, suggesting it may not be as critical as TPIT (Poulin, et al. 1997; Lamolet, et al. 2004). Other proteins, including the orphan nuclear receptor Nur77, also bind to response elements on the Pomc promoter to drive its transcription (Philips, et al. 1997a; Philips, et al. 1997b). Conversely, Pomc transcription is inhibited by the expression of BMPs (Nudi, et al. 2005), (Figure 1.1). While much is known about
the terminal differentiation of these cells, the signals controlling their specification remain unclear. Previous data from our lab show that loss of two Notch effector genes results in premature differentiation of corticotropes, suggesting Notch signaling may play a role in specification of these cells (Himes and Raetzman. 2009a).

The common precursor of somatotropes, lactotropes and thyrotropes begins to express the protein PIT1 at roughly e12.5. Prophet of Pit1 (PROP1) is required for the activation of Pit1 transcription (Nasonkin, et al. 2004). Ames Dwarf mice, which lack functional Prop1, show near complete loss of all cells of the PIT1 lineage (Gage, et al. 1996). PIT1 acts in concert with other molecules to transcriptionally regulate terminal differentiation markers in each of the three cell types. In thyrotropes, which are fully differentiated by e15.5, GATA2 acts synergistically with PIT1 to drive Tshb transcription (Kashiwabara, et al. 2009). Similarly, in lactotropes, which express PRL beginning after birth, PIT1 acts with estrogen receptor and Ets-1 to drive Prl transcription (Bradford, et al. 1996). Thyroid hormone and retinoic acid, acting through their respective receptors, stimulate growth hormone transcription, which is first observed at e17.5 (Palomino, et al. 1998). In addition, Math3, a downstream target of PIT1, is necessary for the expression of growth hormone releasing hormone receptor, which allows somatotropes to respond to stimulatory signals from the hypothalamus (Zhu, et al. 2006; Zhu, et al. 2007), (Figure 1.1).

A gap in knowledge remains regarding how pituitary progenitor cells balance proliferation and differentiation. Additionally, although factors involved in
regulating terminal differentiation and hormone synthesis in each of the pituitary cell types are well characterized, signals necessary for initial cell specification remain largely unclear. Based on previous findings in the pituitary and in other developing organs, we hypothesize that Notch signaling regulates the balance between proliferating progenitors and differentiated cells in the pituitary.

Notch signaling is an evolutionarily conserved pathway that is important for progenitor maintenance and cell fate choices. Signaling occurs between two adjacent cells, one containing the ligand and the other containing the Notch receptors. In mammals, there are two families of ligands, the Delta-like (Dll1, Dll3 and Dll4) and Jagged (Jag1 and Jag2) families, which can bind to the four Notch receptors (Notch1-4). Upon binding, a series of cleavages is triggered, that ultimately results in the release of the Notch intracellular domain (NICD). A member of ADAM metalloprotease family performs the first cleavage, while the second is dependent on gamma secretase. Once the NICD is released, it can then travel into the nucleus. Here it binds to RBPj, an essential Notch cofactor, and other proteins to create a complex, which binds to DNA and activates the transcription of target genes. Canonical Notch targets are members of the Hes and Hey families of basic helix loop helix transcription factors. These factors often repress the transcription of genes necessary for differentiation, thus maintaining the cell as a progenitor (Patten, et al. 2003; Kopan and Ilagan. 2009), (Figure 1.2).

In many developmental contexts, Notch signaling is necessary for the proliferation of progenitor cells, but is not sufficient alone to direct their
proliferation. However, there are select cases in which Notch can promote proliferation, such as in cerebellar granule neuron precursors (Solecki, et al. 2001). Additionally, activated Notch is thought to contribute to cancer initiation in patients with T-ALL (T-cell acute lymphoblastic leukemia), a pediatric leukemia that is often associated with mutations in NOTCH1 (Pancewicz, et al. 2010).

In addition to its role in regulating cell proliferation, Notch signaling has been shown to be important for cell fate decisions. For example, in the intestine Notch signaling biases cells toward an enterocyte fate and away from a secretory fate during adulthood (Stanger, et al. 2005). Additionally, in the inner ear, Notch signaling controls the balance between hair cells and support cells through a mechanism that is thought to involve lateral inhibition. It is hypothesized that hair cells upregulate Notch ligands and activate Notch signaling in neighboring cells. This activation prevents these adjacent cells from becoming hair cells and biases them toward the support cell fate. A reduction in Notch signaling, therefore, results in an increase in hair cell number (Lanford, et al. 1999; Daudet and Lewis. 2005).

It remains unclear how Notch signaling influences cell fate choice and the balance between proliferation and differentiation in the developing pituitary. Expression analysis studies show that the receptors Notch2 and Notch3, the ligands Dll1 and Jag1 and the Notch effectors Hes1 and Hey1 are all present as early as e9.5 in proliferating progenitors. As cells begin to express terminal differentiation makers and migrate towards the developing AL, the expression of these molecules diminishes (Raetzman, et al. 2004; Zhu, et al. 2006). Several
recent studies have used knockout and transgenic mouse models to determine the role this pathway plays in pituitary development. *Hes1* null mice have hypoplastic pituitaries with failure of melanotropes to form (Zhu, et al. 2006; Kita, et al. 2007; Raetzman, et al. 2007). This hypoplastic phenotype is a result of a decrease in pituitary proliferation observed throughout embryogenesis (Monahan, et al. 2009). However, the confounding problem with this study is lack of pituitary specificity in the *Hes1* knockout, making it impossible to differentiate the pituitary from the hypothalamic role of Notch. Additionally, postnatal proliferation is decreased in Ames Dwarf mice, which lack functional *Prop1* (*Prophet of Pit1*), a putative pituitary specific target of Notch signaling (Ward, et al. 2005; Zhu, et al. 2006). In these mice, cells of the PIT1 lineage (lactotropes, thyrotropes and somatotropes) fail to differentiate, and LH and FSH levels are reduced. However, corticotropes are unaffected, indicating their differentiation is likely controlled by an alternative mechanism. These phenotypes are also observed in humans with mutations in the *PROP1* gene (Wu, et al. 1998). Although corticotropes are unaffected in *Prop1*-deficient mice, specification and differentiation of this lineage may also involve Notch signaling because in a global knockout of both *Hes1* and *Prop1*, accelerated differentiation of corticotropes is observed (Himes and Raetzman. 2009b). This phenotype is not observed in either of the single mutants and indicates Notch signaling is required to prevent early differentiation of these cells. Similarly, if hypothalamic-pituitary biased embryonic stem cells are differentiated in culture, Notch needs to be inhibited in order achieve corticotrope fate (Suga, et al. 2011). These data suggest Notch signaling is necessary to
inhibit the differentiation of corticotropes. It remains unclear if Notch signaling is also sufficient to prevent the differentiation of corticotropes and closely related melanotropes, if it can promote proliferation in these cells and the mechanism by which Notch signaling executes these diverse actions. These studies are important to increase our understanding of the role of Notch signaling in the regulation of proliferation and binary cell fate in the pituitary.

In addition to the embryonic wave of pituitary proliferation and differentiation described in detail above, the pituitary undergoes a second wave of proliferation during the first three postnatal weeks in rodents. In rats, during this time, the pituitary expands 5-6 fold in volume (Carbajo-Pérez and Watanabe. 1990). This period of development has only begun to be studied and signaling pathways involved in proliferation and subsequent cell differentiation have not yet been identified. Additionally, the adult pituitary has a remarkable ability to respond to environmental cues and adjust cell number and size according to need. For example, after adrenalectomy or gonadectomy, the pituitary proliferates due to lack of negative feedback from target organs (Nolan, et al. 2004; Nolan and Levy. 2006). Similarly, during pregnancy, the pituitary expands in anticipation of the increased need for prolactin during lactation (Yin and Arita. 2000; Carretero, et al. 2003).

Although postnatal proliferation has been characterized (Carbajo-Pérez and Watanabe. 1990; Kominami, et al. 2003; Gremeaux, et al. 2012), the identity of the proliferating cell type (s) remains unclear. Differentiated, hormone-producing cells have the ability to proliferate during these periods, but they do so
at a very low rate, making them unlikely to be the sole contributor to pituitary growth (Taniguchi, et al. 2000; Taniguchi, et al. 2001; Taniguchi, et al. 2002a; Taniguchi, et al. 2002b). Pituitary progenitors are present at this stage, found lining the pituitary cleft, a remnant of the lumen of RP, and scattered throughout the AL. Although SOX2 expression decreases in cells in the parenchyma of the AL as the animal ages, progenitor cells remain surrounding the cleft throughout the life of the animal and maintain the ability to generate all pituitary endocrine cell types (Fauquier, et al. 2008; Rizzoti, et al. 2013; Andoniadou, et al. 2013). Additionally, it is hypothesized that a transit amplifying population is derived from these progenitor cells. We hypothesize that these transit amplifying and progenitor populations contribute to postnatal pituitary expansion and may be activated in response to environmental cues.

Because these postnatal and adult progenitors face similar fate choices as their embryonic counterparts, it is likely that similar signaling pathways will be involved. This progenitor population was shown to be enriched in Notch signaling components in postnatal mice and inhibition of Notch signaling in dissociated pituitary cells or in pituitary cell aggregates results in a decrease in the size and proliferation of the progenitor pool. Increased progenitor proliferation and number are observed when these cells are treated with Notch ligands (Chen, et al. 2006; Tando, et al. 2013). However, neither the mechanism accounting for these observations, nor the consequences of a reduction in the progenitor population in vivo, were investigated. Based on these findings and its known role in the pituitary during embryogenesis, we hypothesize Notch signaling may act to
maintain progenitor cells in the postnatal and adult pituitary and thus influences pituitary proliferation and cell fate specification.

Improper pituitary cellular proliferation or differentiation during either the embryonic or postnatal period can result in multiple pituitary disorders, including combined pituitary hormone deficiency and adenoma formation. It is, therefore, critical, to understand mechanisms controlling these processes. We hypothesize that Notch controls binary cell fate choice and proliferation during all stages of pituitary development. The studies outlined below aim to determine: 1) the role of Notch signaling in embryonic corticotrope development using a pituitary-specific gain- and loss-of-function approach, 2) if Notch is necessary for proliferation, progenitor maintenance and cell fate choice during postnatal pituitary expansion and 3) the identity of Notch targets that are important during postnatal pituitary development.
REFERENCES


Ericson, J., Norlin, S., Jessell, T.M., Edlund, T., 1998. Integrated FGF and BMP Signaling Controls the Progression of Progenitor Cell Differentiation and the


Nudi, M., Ouimette, J., Drouin, J., 2005. Bone Morphogenetic Protein (Smad)-Mediated Repression of Proopiomelanocortin Transcription by Interference with Pitx/Tpit Activity. Molecular endocrinology (Baltimore, Md) 19, 1329-42.


Figure 1.1. Schematic of pituitary development. The anterior and intermediate lobes of the pituitary develop from the most anterior portion of the neural ridge, which expresses Foxg1. The posterior lobe (P) develops from the neural tissue that is just adjacent to this region. At e9.5, induction of the pituitary relies on expression of Wnt5a, BMP-4, and FGF8/10/18 in the VD and SHH in the oral ectoderm. At this age, RP is marked by the expression of the transcription factor LHX3. By e11.5, RP pinches off from the underlying oral ectoderm to form a closed pouch. By e12.5, the emergence of differentiated cell types is evident, as shown by expression Pomc mRNA in corticotropes. Pit1+ cells, which are precursors of lactotropes, thyrotropes and somatotropes, and SF1+ cells, which are precursors to gonadotropes, are observed by e13.5. Melanotropes in the IL are evident by e16.5 and GH and TSHβ are expressed in the AL by e17.5 (A). The differentiation of corticotropes and melanotropes rely on the expression of Tbx19, with corticotropes also requiring expression of NeuroD1 for Pomc transcription. Expression of Pit1 is reliant on Prop1, whereas each cell type of the Pit1 lineage requires additional transcription factors for hormone expression (ER for lactotropes, TR for somatotropes and Gata2 for somatotropes). Gonadotropes develop from SF1+ precursors and require Egr1 for hormone production (B). RP=Rathke’s pouch, VD=Ventral diencephalon, T=Thyrotropes, S=Somatotropes, L=Lactotropes, C=Corticotropes, M=Melanotropes. (Adapted from Zhu et al, 2007).
Figure 1.2. Schematic of Notch signaling. Notch signaling occurs between two adjacent cells, one that contains a ligand of the Delta-like and/or Jagged families and the other that contains the Notch receptor. Upon ligand binding, the intracellular domain of Notch is cleaved by gamma secretase. It can then translocate into the nucleus where it binds several cofactors. These cofactors include RBPj, which is the cofactor that binds directly to DNA and Mastermind-like, which serves as a scaffold for the recruitment of other coactivators. Activation of this cascade allows the NICD-containing complex to drive the transcription of downstream targets of the Hes and Hey families of transcriptional repressors. Dll=Delta-like.
CHAPTER 2: Persistent Expression of Activated Notch inhibits corticotrope and melanotrope differentiation and results in dysfunction of the HPA axis\(^1\).

ABSTRACT

The hypothalamic-pituitary-adrenal (HPA) axis is an important regulator of energy balance, immune function and the body’s response to stress. Signaling networks governing the initial specification of corticotropes, a major component of this axis, are not fully understood. Loss of function studies indicate that Notch signaling may be necessary to repress premature differentiation of corticotropes and to promote proliferation of pituitary progenitors. To elucidate whether Notch signaling must be suppressed in order for corticotrope differentiation to proceed and whether Notch signaling is sufficient to promote corticotrope proliferation, we examined the effects of persistent Notch expression in Pomc lineage cells. We show that constitutive activation of the Notch cascade inhibits the differentiation of both corticotropes and melanotropes and results in the suppression of transcription factors required for Pomc expression. Furthermore, persistent Notch signaling traps cells in the intermediate lobe of the pituitary in a progenitor state, but has no effect on pituitary proliferation. Undifferentiated cells are eliminated in the first two postnatal weeks in these mice, resulting in a modest increase in CRH expression in the paraventricular nucleus, hypoplastic adrenal glands and decreased stress-induced corticosterone levels. Taken together, these findings

\(^1\) This chapter appeared in its entirety in *Developmental Biology* and is referred to later in this dissertation as “Goldberg et al., 2011”. Goldberg, L.B., Aujla, P.K., Raetzman, L.T., 2011. Persistent Expression of Activated Notch Inhibits Corticotrope and Melanotrope Differentiation and Results in Dysfunction of the HPA Axis. Dev Biol 358, 23-32. This article is reprinted with the permission of the publisher and is available from http://www.elsevier.com and using DOI: 10.1016/j.ydbio.2011.07.004
show that Notch signaling is sufficient to prevent corticotrope and melanotrope differentiation, resulting in dysregulation of the HPA axis.

INTRODUCTION

The hypothalamic-pituitary-adrenal (HPA) axis is critical to mediating the body’s response to stress. It also functions to regulate many important homeostatic processes, including immune response and energy balance. Stressors, either immune or environmental, activate the release of corticotropin releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus to the anterior pituitary through the hypothalamo-hypophyseal portal system. CRH release stimulates the anterior pituitary to synthesize and release adrenocorticotropic hormone (ACTH), which acts on the adrenal glands to trigger the release of cortisol (corticosterone in mice) (Garren, 1968; Vale, et al. 1981). Increased cortisol release results in a number of different physiological effects, including an increase in glucose production and mobilization and an anti-inflammatory response (Munck, et al. 1984). In addition to increased glucose release, cortisol also exerts negative feedback on both the hypothalamus and pituitary, inhibiting further release of CRH and ACTH (Swanson, Simmons. 1989). Disruption in proper development or function at any level of the HPA axis can result in multiple disorders, including Cushing’s syndrome and adrenal insufficiency, which are characterized by increased and decreased levels of cortisol, respectively (Drouin, et al. 2007).
The pituitary, a crucial component of the HPA axis, develops from an invagination of the oral ectoderm at embryonic day (e) 8.5 in mice. By e11.5, the developing pituitary pinches off from the underlying oral ectoderm, forming the structure known as Rathke’s pouch (RP). RP contains all the progenitor cells that are necessary for formation of the anterior lobe (AL) and intermediate lobe (IL) of the pituitary (Burrows, et al. 1999; Rizzoti, Lovell-Badge. 2005). The AL contains five hormone producing cell types, which include thyrotropes, lactotropes, gonadotropes, corticotropes, somatotropes, and lactotropes. The IL houses a sixth hormone cell type, the melanotropes. Current models propose that pituitary hormone producing cells are specified such that each cell type differentiates during a discrete time during embryonic development (Pope, et al. 2006). Furthermore, it is widely accepted that cells exiting the cell cycle at the same time migrate to the same location in the pituitary (Dasen, Rosenfeld. 2001; Wagner, Thomas. 2007). This synchronized cell specification and migration is thought to be coordinated by opposing gradients of bone morphogenetic proteins (BMPs) in the underlying oral ectoderm and fibroblast growth factors (FGFs) in the ventral diencephalon (Ericson, et al. 1998; Treier, et al. 1998). However, recent evidence indicates that each AL hormone cell type exits the cell cycle at the same time, with the majority of cells doing so between e11.5 and e13.5. Furthermore, there is no apparent correlation between the timing of cell specification and cell placement within the gland (Davis, et al. 2011). Based on these observations, it remains unclear how undifferentiated progenitor cells...
within the same region of the AL, exposed to the same chemical gradients, are specified to become different cell types.

Although all AL hormone cell types exit the cell cycle at the same time, corticotropes are the first to express hormone beginning at e12.5 (Japón, et al. 1994). Melanotropes, found in the IL, exit the cell cycle after AL hormone producing cells and express hormone beginning at e16.5 (Davis, et al. 2011). Terminal differentiation of corticotropes and melanotropes is characterized by the expression of pro-opiomelanocortin (POMC), a precursor of both ACTH, which is released by corticotropes, and melanocyte stimulating hormone (αMSH), which is produced by melanotropes.

Several studies have identified multiple transcription factors that activate or inhibit expression of POMC in the pituitary. These include TPIT (TBX19), (Lamolet, et al. 2001; Liu, et al. 2001), NEUROD1 (Poulin, et al. 1997; Lamolet, et al. 2004), Nur77 (Philips, et al. 1997a; Philips, et al. 1997b) and BMPs (Nudi, et al. 2005). While these factors are known to regulate Pomc transcription and thus the terminal differentiation of corticotropes and melanotropes, it remains unclear which signals are involved in the specification of these cells.

We hypothesize that the Notch signaling pathway, an essential player in progenitor maintenance and cell fate determination in many tissues, may play a critical role in the specification of corticotropes and melanotropes. The Notch ligands Delta-like1 and Jagged1, the Notch2 and Notch3 receptors, as well as downstream Notch effectors of the Hes and Hey families of genes, are present during development in proliferating progenitors in RP. As cells differentiate and
migrate towards the developing AL, the expression of these molecules is suppressed (Raetzman, et al. 2004; Zhu, et al. 2006). Additionally, the Notch1 and Notch2 receptors, as well as Hes and Hey transcription factors, are present in pituitary stem cells (Chen, et al. 2005; Chen, et al. 2006).

Studies have shown that loss of function manipulations of the Notch signaling pathway result in defects in corticotrope and melanotrope differentiation. In mice lacking Hes1, a canonical Notch target, Tpit mRNA expression was found to be increased (Zhu, et al. 2006), suggesting that Notch may be necessary to prevent early expression of this transcription factor. When both Prop1, a pituitary specific Notch target, and Hes1 are lost, robust premature differentiation of corticotropes is observed (Himes, Raetzman. 2009). A similar, but more modest, acceleration of Pomc expression is seen in a pituitary-specific knockout of Rbpj, the primary mediator of Notch signaling (Zhu, et al. 2006). Taken together, these results suggest Notch signaling is necessary to prevent differentiation of corticotropes and may act as a negative regulator of early corticotrope specification. We hypothesize that Notch signaling is sufficient to prevent the differentiation of corticotropes and melanotropes and that Notch expression needs to be extinguished for cell differentiation to proceed.

In order to address this hypothesis, we examined the effects of persistent expression of activated Notch1 in POMC-expressing cells. We find that Notch signaling is sufficient to prevent differentiation of these cells, coincident with the suppression of the transcription factors Tpit and Neurod1. Additionally, we show that these undifferentiated cells maintain progenitor qualities but are eliminated
during the first two postnatal weeks. The loss of POMC expression in the pituitary results in severely hypoplastic adrenal glands and decreased stress-induced corticosterone levels. Our results indicate that Notch signaling is both necessary and sufficient to repress corticotrope differentiation and that alterations in Notch signaling could result in dysfunction of the HPA axis.

MATERIALS AND METHODS

Mice

Rosa<sup>Notch</sup> floxed mice (Murtaugh, et al. 2003) were purchased from Jackson Laboratories (Bar Harbor, ME). A breeding colony was established and progeny were bred to Pomc Cre mice (Balthasar, et al. 2004), also purchased from Jackson Laboratories (Bar Harbor, ME). The resulting progeny all possess one Rosa<sup>Notch</sup> floxed allele, with half also expressing Cre recombinase. To genotype the mice, tail biopsies were obtained and DNA was extracted using a salt-out method. PCR for the Pomc and Cre alleles was performed as previously described (Himes, Raetzman. 2009). PCR for the Rosa<sup>Notch</sup> floxed allele was performed in a similar manner with the following exceptions. The primers used to detect this allele were 5'-AAA GTC GCT CTG AGT TGT TAT-3', 5'-TAA GCC TGC CCA GAA GAC T-3' and 5'-GAA AGA CCG CGA AGA GTT T G-3'. The samples underwent 35 cycles of denaturing at 95 C for 30 sec, annealing at 55 C for 30 sec, and elongation at 72 C for 60 sec, followed by 72 C for 5 min. All animals were housed in a facility with a 12 hour light-dark cycle and were maintained in accordance with the University of Illinois at Urbana-Champaign.
Institutional Animal Care and Use Committee.

**Immunohistochemistry**

Mice were sacrificed at e16.5, p1, and p32 and embryos and pituitaries were fixed in 3.7% formaldehyde diluted in phosphate buffered saline (PBS). Samples were dehydrated through a series of graded ethanol, embedded coronally in paraffin, sectioned to a thickness of 6 microns and mounted on charged slides. For frozen section preparation used to detect CRH in the hypothalamus, p32 control and Tg brains were snap frozen in 2-methylbutane, cryoprotected overnight at 4°C in 30% sucrose diluted in PBS, and sectioned at 14µm.

Paraffin sections were deparaffinized, rehydrated and washed in PBS. Samples were then boiled in hot citrate solution (10μM citrate, pH6) for 10 minutes and cooled for 10 minutes (for all antibodies except POMC and CRH). Following antigen retrieval, samples were blocked in a solution containing 5% normal donkey serum diluted in immunohistochemical block which contains 3% bovine serum albumin and 0.5% TritonX-100 diluted in PBS. Frozen sections were fixed in 4% PFA for 30 mins, washed in PBS prior to application of blocking solution. For all immunohistochemistry, blocking was followed by an overnight incubation at 4°C with primary antibody diluted in immunohistochemical block. Primary antibodies used were raised against the following peptides: POMC (1:300, Dako, Carpinteria, CA), Sox2 (1:750, Millipore, Billerica, MA), Pax7 (1:500, Developmental Studies Hybridoma Bank, Iowa City, IA), ki67 (1:100, Dako, Carpinteria, CA), phospho-histone-H3 (1:500 Upstate Cell Signaling...
Solutions, Lake Placid, NY) and CRH (1:1000, Millipore, Billerica, MA, USA).

Slides were then incubated with biotin-conjugated rat (ki67), mouse (Pax7) or rabbit (POMC, Sox2, phospho-histone-H3) secondary antibody diluted in immunohistochemical block for one half hour at room temperature. This was followed by a series of washes and a thirty-minute incubation with a streptavidin-conjugated cy3 or dylight488 fluorophore. Secondary and streptavidin-conjugated antibodies were purchased from Jackson ImmunoResearch (West Grove, PA) and were used at a concentration of 1:200. Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:1000, Sigma, St. Louis, MO) and visualized at 100x, 200x, or 400x magnification using a Leica DM2560 microscope. Photographs were taken using a Retiga 2000R color camera (Q-Imaging, Surrey, Canada) and acquired using Q-Capture Pro software (Q-Imaging). Images were processed using Adobe Photoshop CS4 (San José, CA).

Quantitative Reverse Transcriptase PCR

RNA was isolated from embryonic and postnatal whole pituitaries using an RNAqueous micro kit (Ambion, Austin, TX) as per manufacturers protocol. For p32 pituitaries, 0.5µg of RNA was synthesized into cDNA using the ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA). For e16.5 and p1 pituitaries, RNA was isolated from individual pituitaries of each genotype and the total RNA from each pituitary was converted into cDNA. A no enzyme control was also prepared and used as a negative control. For quantitative RT-PCR 0.2µL of cDNA from p32 and p1 pituitaries and 0.5µL from e16.5 pituitaries was amplified using gene-specific primers and SYBR green mix.
(Bio-Rad Laboratories, Hercules, CA) on a Bio-Rad MyIQ real-time PCR machine. Data were analyzed with the change in cycle threshold (ΔCt) value method. Specifically, for each sample, the mean Ct of the gene of interest was calculated as an average of the duplicates of that sample. This normalized mean was subtracted from the mean Ct for Gapdh to obtain the ΔCt. The ΔΔCt was then calculated as the difference between the ΔCt for each Tg and each control mouse. The relative fold change of Tg mice as compared to control mice was calculated as $2^{-\Delta\Delta Ct}$. The error bars represent the standard error of the mean of the relative fold change. Statistical significance was determined using Student’s t test.

Gene specific primers for real time RT-PCR include: Gapdh forward, GGT GAG GCC GGT GCT GAG TAT G; Gapdh reverse, GAC CCG TTT GGC TCC ACC CTT C; Hes1 forward, CTC GCT CAC TTC GGA CTC; Hes1 reverse, GTG GGC TAG GGA CTT TAC; Hey1 forward, CAC GCC ACT ATG CTC AAT; Hey1 reverse, CCT TCA CCT CAC TGC TCT G; Hey2 forward, GAT TCC GAG AGT GCT TGA C; Hey2 reverse, AGG TGC TGA GAT GAG AGA C; Heyl forward, GGA ACA ACA GAG AAT GAA C; Heyl reverse, CAG CAG TAG TGA GTA ACC; Pomc forward, GTT ACG GTG GCT TCA TGA CCT C; Pomc reverse, CGC GTT CTT GAT GAG CAC TTT TCT ATC AAA TTC ACT GA; Neurod1 forward, GCC GCCA GTG GCT TCA TGA CCT C; Neurod1 reverse, AGC TTT TCT ATC AAA TTC ACT GA; Mash1 forward, TGG ACT TTG GAA GCA GGA TGG; Mash1 reverse, TGA CGT CGT TGG CGA GAA ACA; Myc forward, TGA CCT AAC
TCG AGG AGG AGC TGG AAT C; *Myc* reverse, AAG TTT GAG GCA GTT AAA ATT ATG GCT GAA GC.

Reverse Transcriptase PCR

RNA and cDNA form p5, p7 and p9 pituitaries were prepared in the same way as described above with the exception that 0.33µL of cDNA was amplified. Amplification was performed using *Pax7* specific primers on a C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Amplification was visualized using an ethidium bromide stained gel. Sequences of primers used: *Pax7* forward, GCA CAG AGG ACC AAG CTC AC; *Pax7* Reverse TGG TGG TGG GGT AGG TAG AG.

Corticosterone Assay and Adrenal Collection

p32 mice were subjected to one hour of restraint stress in a ventilated 50mL conical tube beginning at 1500h. At 1600h, mice were sacrificed using CO$_2$. Blood was collected by cardiac puncture and allowed to clot for 30 minutes in EDTA-coated tubes. Blood was spun down and plasma was collected and stored at -20°C until needed. A corticosterone Enzyme Immunoassay kit (Assay Designs, Ann Arbor, MI) was used to measure plasma corticosterone levels as per manufacturers protocol. All samples were run in duplicate and statistical significance was determined using Student’s *t* test. Adrenals were dissected from p32 male and female mice. They were fixed overnight in 3.7% formaldehyde diluted in PBS, washed in PBS and photographed.
RESULTS

NICD expression in melanotropes and corticotropes activates Notch target genes

To determine the effect of aberrant Notch activity during corticotrope and melanotrope development, we generated a mouse model with persistent expression of the activated Notch1 intracellular domain (NICD) in POMC-expressing cells. This mouse was generated from the mating of a Rosa\textsuperscript{Notch}\textsuperscript{floxed} mouse (Murtaugh, et al. 2003) to a mouse expressing Cre recombinase under the control of the Pomc promoter (Balthasar, et al. 2004). POMC is expressed in corticotropes in the AL and in melanotropes in the IL. Additionally, POMC is produced by hypothalamic neurons in the arcuate nucleus. However, these neurons do not innervate pituitary melanotropes or corticotropes and are formed through different mechanisms than pituitary cells (McNay, et al. 2006)(Oertel, et al. 1982; Léránth, et al. 1983; Mezey, et al. 1984; Kawano, Daikoku. 1987; Goudreau, et al. 1995). In the absence of Cre recombinase, a stop codon flanked by loxP sites prevents the expression of the single-copy NICD construct. However, when Cre recombinase is active, the stop codon is excised allowing for the constitutive expression of NICD in Pomc-expressing cells (Figure 2.1A). Although Notch1 is not normally expressed during pituitary development, studies have shown that the intracellular domains of Notch1 and Notch2 can have similar transcriptional activity (Kraman, McCright. 2005). Furthermore, Notch1 is expressed in the adult stem cell population (Chen, et al. 2005; Chen, et al. 2006), indicating it may play a role in cell fate choice or maintaining progenitor cells in the adult pituitary. Therefore, it is of interest to
determine the effects of persistent Notch1 expression in pituitary cell specification during development and in postnatal progenitor maintenance.

Quantitative RT-PCR for Hes1, Hey1, Hey2 and Heyl was performed on Rosa\textsuperscript{Notch/+} (control) and Rosa\textsuperscript{Notch/+}; Pomc-Cre (Tg) pituitaries at e16.5 to determine if NICD overexpression results in an increase in any of the canonical Notch effector genes. The age e16.5 was chosen to begin analysis since it is the earliest developmental stage at which POMC expression is present in both corticotropes and melanotropes. Our results show that Hes1 mRNA levels are unchanged between the two experimental groups. However, Hey1, Hey2, and Heyl are all significantly increased in Tg pituitaries as compared to their control counterparts (Figure 2.1B). These results indicate that overexpression of Notch in pituitary melanotropes and corticotropes is sufficient to produce a modest change in Notch effector mRNA levels.

Persistent expression of activated NICD is sufficient to prevent corticotrope and melanotrope terminal differentiation

Notch signaling is known to regulate the balance between proliferating progenitors and differentiated cells in many endocrine organs. Therefore, aberrant Notch signaling could possibly alter the size or morphology of the developing pituitary. To detect if any such morphological changes are present in Tg mice, control and Tg pituitary sections were stained with hematoxylin and eosin. At e16.5, the morphology and size of the control (Figure 2.2A) and Tg (Figure 2.2B) pituitaries appear similar, with all three lobes present in both experimental groups. The same is true at postnatal day (p) 1, when pituitaries of
control (Figure 2.2E) and Tg (Figure 2.2F) mice are histologically indistinguishable. Since Notch signaling is also implicated in cell differentiation in many tissues, we used immunohistochemistry to examine whether POMC expression is affected by persistently activated Notch expression at e16.5 and p1. At e16.5, control mice have POMC-positive cells in the IL and scattered throughout the AL (Figure 2.2C). At this same age, Tg mice have very few POMC-positive cells in either the AL or IL. (Figure 2.2D), indicating their differentiation is prevented by expression of NICD. This phenotype persists through p1, when almost no cells in the IL or AL of Tg mice are POMC-positive (Figure 2.2H), compared to the control (Figure 2.2G). Pomc mRNA levels of control and Tg e16.5 pituitaries were measured using quantitative RT-PCR to determine if this transcript is altered by persistent NICD expression. Similar to immunohistochemical observations, Tg mice have significantly lower levels of Pomc mRNA as compared to control littermates (Figure 2.2I). Taken together, these data show that constitutive NICD expression in melanotropes and corticotropes is sufficient to inhibit their differentiation and imply that it is critical that Notch signaling be suppressed in order for corticotrope and melanotrope differentiation to proceed.

Activated Notch inhibits the transcription of factors necessary for POMC expression

To better elucidate the mechanism by which Notch inhibits the differentiation of corticotropes and melanotropes, quantitative RT-PCR was used to examine the mRNA levels of the transcription factors Tpit, Neurod1 and Mash1
in control and Tg mice at e16.5. Tpit and Neurod1 have been shown to play a role in the activation of Pomc transcription, whereas Mash1 appears to be restricted to Pomc containing cells, although it is not required for Pomc transcription (Poulin, et al. 1997; Lamolet, et al. 2001; Liu, et al. 2001; Pulichino, et al. 2003a; Pulichino, et al. 2003b; Lamolet, et al. 2004; McNay, et al. 2006). Tpit, NeuroD1 and Mash1 all show significantly lower mRNA levels in Tg mice as compared to their control counterparts, with Tpit showing the strongest suppression (Figure 2.3). Therefore, Notch signaling components may directly or indirectly affect the expression of factors important for terminal differentiation of corticotropes and melanotropes.

Undifferentiated IL cells remain SOX2-positive progenitors

Given that IL cells fail to differentiate when activated NICD is constitutively expressed, we hypothesized that these cells may retain markers of pituitary progenitors, such as SOX2 (Fauquier, et al. 2008). In control mice, SOX2-positive cells are seen in the IL in a thin layer lining the lumen of the pituitary (Figure 2.4, A, C, E and G). Conversely, in Tg mice, nearly every cell in the IL is positive for SOX2, indicating these cells maintain progenitor cell qualities (Figure 2.4, B, D, F and H). Pax7 is a putative marker of intermediate pituitary progenitors (Hosoyama, et al. 2010) and is expressed throughout the IL in control mice at p1 (Figure 2.4I), however a thin layer of cells lining the pituitary cleft are mostly Pax7-negative (Figure 2.4 K, M, O). Based on localization, they likely constitute SOX2-positive cells. In Tg mice, Pax7 is also restricted to the IL (Figure 2.4J). However, nearly every cell in the IL, including those lining the cleft,
expresses Pax7 (Figure 2.4, L, N, P). Taken together, these findings indicate that constitutive NICD expression results in an increased progenitor population in the IL.

Activated Notch leads to elimination of undifferentiated melanotropes and corticotropes

Morphology and size of adolescent (p32) control and Tg pituitaries were examined using hematoxylin and eosin in order to determine if persistent Notch expression affects pituitary morphology at later ages. In control pituitaries, a prominent IL is observed surrounding the PL (Figure 2.5A). In contrast, the IL of Tg mice is reduced to a single-cell thick layer surrounding the PL (Figure 2.5B). The AL and PL look similar in both experimental groups. POMC expression was analyzed at p32 to determine if the remaining cells in the IL of adolescent Tg mice express terminal differentiation markers. In control mice, nearly every cell in the IL expresses POMC (Figure 2.5C). However, in Tg mice, only a handful of the residual IL cells are POMC-positive (Figure 2.5D). In addition, very few POMC immunoreactive cells are observed in the AL of Tg mice (Figure 2.5F), as compared to control mice (Figure 2.5E). The near complete loss of POMC-expressing cells was confirmed by quantitative RT-PCR for Pomc, which shows dramatically lower levels of Pomc mRNA in Tg mice than in control littermates at p32 (Figure 2.5G). We also observe a reduction in Crhr1 mRNA in Tg mice, further indicating there is a loss of both melanotropes and corticotropes (data not shown). To determine if the corticotropes and melanotropes are able to regenerate in older mice, pituitary sections from four-month-old control and Tg
mice were stained for POMC. Similarly, almost no POMC expression was observed in the AL or IL of Tg mice (data not shown). These results suggest that expression of activated Notch results in the elimination of undifferentiated melanotropes and corticotropes.

In order to better elucidate the age at which the elimination of these cells occurs, TUNEL staining was performed at e16.5 and p1. No differences were observed between control and Tg pituitaries at either age (data not shown). This indicates that cell death is not initiated until later postnatal development in Tg mice. In order to determine the developmental window where IL cells are eliminated, Pax7 RT-PCR was performed on p5, p7, and p9 control and Tg pituitaries (Figure 2.5H). Since previous results suggest Pax7 is maintained in undifferentiated IL cells in Tg mice (see Figure 2.2J), this approach was an effective method to determine the age at which IL cells are no longer present. At p5, pituitaries from both control and Tg mice express roughly equal levels of Pax7, suggesting NICD-containing cells are still present at this age. By p7, Pax7 levels in Tg mice appear to be suppressed as compared to control mice. In one p7 Tg mouse, Pax7 levels are visibly reduced as compared to control, whereas another Tg animal shows near complete loss of Pax7 pituitary mRNA. At p9, all Tg pituitaries examined contain no detectable levels of Pax7 mRNA, whereas control pituitaries still maintain Pax7 expression. These findings show that aberrant expression of NICD results in elimination of NICD-containing cells within the first two postnatal weeks.
Persistently activated NICD expression in POMC-expressing cells results in HPA axis dysfunction

The effects of NICD expression in corticotropes may be reflected in alterations throughout the HPA axis. Development and function of the adrenal cortex relies heavily on the release of ACTH from the pituitary (Estivariz, et al. 1982; Simpson, Waterman. 1988). Therefore, adrenal glands were compared between control and Tg adolescent mice at p32. Control mice have visibly larger adrenal glands than Tg counterparts in both sexes (Figure 2.5I). To determine the functional capabilities of the hypoplastic adrenals in Tg mice, stress-induced corticosterone levels were measured. Significant decreases in levels of corticosterone are observed in both male and female Tg mice as compared to control counterparts (Figure 2.5J).

Under normal conditions, both corticosterone released from the adrenal gland and ACTH released from the pituitary can influence CRH release at the level of the hypothalamus (Swanson, Simmons. 1989). Given that both corticosterone levels and ACTH-positive cells are decreased in Tg mice, we would expect that CRH levels in the hypothalamus would be increased as a result of positive feedback. To determine differences in CRH expression between Tg and control male mice at p32, immunohistochemical expression of CRH was examined in the PVN. Tg mice display a slight increase in CRH immunoreactivity in the medial parvocellular portion of the PVN (PaMP; Figure 2.6A), a region known to be highly responsive to stress (Renard, et al. 2010). As expected, without colchicine pretreatment to inhibit axonal transport of peptides, CRH-
positive cell bodies in control animals are hardly detectable, with some immunopositive processes present. In contrast, Tg animals display immunoreactive CRH-positive cell bodies as well as neuronal processes, indicating an increased level of CRH in this region (Figure 2.6B). These data are consistent with CRH localization in Pomc knockout mice (Yaswen, et al. 1999; Smart, et al. 2007) and, taken together with the decreased levels of corticosterone, show that Notch-induced loss of POMC expression in the pituitary leads to subsequent dysfunction of the HPA axis.

**Activated NICD in melanotropes and corticotropes is not sufficient to promote their proliferation**

Because Notch is known to promote proliferation in many cell types (Solecki, et al. 2001; Collesi, et al. 2008; Zhang, et al. 2008), we hypothesized it may have a similar role in corticotropes and melanotropes. We, therefore, examined the expression of proliferation markers in p1 control and Tg pituitaries. Ki67, a marker of all stages of the cell cycle, is observed throughout the AL, IL and PL in control mice (Figure 2.7A). No difference in expression pattern was observed in Tg mice (Figure 2.7B), indicating overall proliferation in Pomc-expressing cells is unaffected by the constitutive expression of NICD. Phospho-histone-H3 staining was also performed in order to determine if mitosis is altered in cells persistently expressing NICD. Again, no visible changes in expression were observed between control (Figure 2.7C) and Tg (Figure 2.7D) pituitaries at p1. Histological observations were confirmed by quantitative RT-PCR analysis of control and Tg p1 pituitaries, which showed no difference in Myc mRNA levels
between the two experimental groups (Figure 2.7E). These results indicate that activated NICD is insufficient to promote proliferation in undifferentiated Pomc-expressing cells.

**DISCUSSION**

Disruption in normal corticotrope development or function can result in various disorders, including isolated ACTH deficiency and Cushing's syndrome (Drouin, et al. 2007). Signals controlling the commitment and specification of corticotropes are not well understood, but the Notch signaling pathway has been implicated in these processes. We have previously demonstrated that global loss of *Hes1* and *Prop1*, two Notch effector genes, results in premature differentiation of corticotropes, indicating this pathway is necessary to prevent early differentiation of these cells (Himes, Raetzman. 2009). Given this finding, we hypothesized that Notch signaling is also sufficient to inhibit the differentiation of both corticotropes and closely related melanotropes. Using a mouse model in which activated Notch is persistently expressed in these two cell types, we were able to confirm this hypothesis.

As in many developing tissues, Notch signaling components are found in RP progenitors and in the adult progenitor population but are excluded from differentiated cells (Raetzman, et al. 2004; Zhu, et al. 2006). The presumptive pituitary progenitor population also contains the transcription factor SOX2, which is thought to be important in maintaining pluripotency and inhibiting cell differentiation (Episkopou. 2005; Masui, et al. 2007). SOX2 is expressed in
isolated adult murine pituitary progenitors and is mainly localized to a single cell layer lining either side of the pituitary cleft in the adult (Rizzoti. 2010). In cochlear development, suppression of Notch signaling results in a decrease in the expression of SOX2 and in increase in differentiated cells (Dabdoub, et al. 2008). It is possible that SOX2 is a direct target of the Notch signaling cascade because luciferase reporter assays have shown that NICD has the ability to activate transcription of the SOX2 promoter (Ehm, et al. 2010). It is, therefore, not surprising that we observe an increase in SOX2 immunoreactivity in pituitary cells that contain activated Notch. This increase in SOX2 may correlate with an increase in multipotent pituitary progenitor cells, and indicates that Notch signaling acts in a similar fashion in the pituitary as it does in other developing systems.

We observed that inappropriately specified cells in the pituitary expressing NICD do not reenter the cell cycle and are eliminated during the first two postnatal weeks. Persistent expression of activated Notch has been shown to induce apoptosis in multiple cell types, including intestinal and neural progenitors (Yang, et al. 2004; Fre, et al. 2005). Although we cannot rule out a role for activated Notch in inducing apoptosis, it is likely that these cells are eliminated because they are undifferentiated. Ames dwarf mice, which lack functional Prop1, have extensive apoptosis in aberrantly undifferentiated cells in the pituitary at p1 and p8 (Ward, et al. 2005). This data supports the hypothesis that undifferentiated pituitary cells may be more prone to cell death, particularly

While Notch signaling in early development is known to influence the balance between proliferating progenitors and differentiated cells, it also directs cell fate determination at later stages of development in many systems. For example, Notch promotes cerebellar granule neuron precursor proliferation (Solecki, et al. 2001), but also promotes radial glia differentiation and inhibits granule neuron differentiation at later stages of development (Patten, et al. 2003). A similar dual role of Notch is seen in the intestine where Notch signaling inhibits stem cell differentiation during embryogenesis but biases cells toward an enterocyte fate and away from a secretory fate during adulthood (Stanger, et al. 2005). We found that that NICD is not sufficient to convert melanotropes and corticotropes to an alternate cell fate but instead traps them in a progenitor fate. This is especially clear in the IL where NICD-containing cells do not adopt another fate, but rather retain progenitor-like properties. However, since we are altering Notch signaling only after cells already committed to become melanotropes and corticotropes, it is possible that Notch influences cell fate at a different developmental stage as it does in other tissues discussed above.

Through both gain and loss of function studies, it is clear that Notch can influence terminal differentiation of Pomc containing cells (Zhu, et al. 2006; Dutta, et al. 2008; Himes, Raetzman. 2009). Molecules required for Pomc transcription, and therefore for the terminal differentiation of corticotropes and melanotropes, have been well characterized. These factors include TPIT (Liu, et al. 2001;
Pulichino, et al. 2003b) and NEUROD1 (Poulin, et al. 1997; Lamolet, et al. 2004). In contrast, BMPs have been shown to inhibit the expression of Pomc in vitro (Nudi, et al. 2005). While these factors are known to regulate Pomc transcription and thus the terminal differentiation of corticotropes and melanotropes, it remains unclear which signals are involved in the specification of these cells. Lineage specification is likely controlled in part by extrinsic signals, such as morphogens expressed in the oral ectoderm and ventral diencephalon surrounding the developing pituitary. However, cells located in the same region during development do not necessarily develop into the same cell type (Davis, et al. 2011), suggesting intrinsic properties are also likely involved. We hypothesize that Notch signaling may be one such intrinsic factor. We show that persistently expressing NICD in corticotropes and melanotropes results in suppression of Tpit and Neurod1, indicating their expression may be under the control of the Notch signaling cascade. It is also possible that the suppression of these transcription factors is a result of the progenitor-like nature of the cells, which are ectopically expressing SOX2, and is not a result of their direct regulation by NICD. Future studies will be necessary in order to elucidate if and how Notch signaling directly regulates these molecules. Taken together with the fact that Prop1, a direct transcriptional target of Notch signaling, is required for PIT1 expression and the subsequent differentiation of lactotropes, somatotropes, and thyrotropes (Gage, et al. 1996a; Gage, et al. 1996b; Zhu, et al. 2006), we propose a model by which the Notch signaling pathway regulates the choice between precursors of the corticotrope and PIT1 lineages during early pituitary cell specification (Figure 2.8)
This study is the first to show that Notch signaling is sufficient to inhibit melanotrope and corticotrope differentiation, resulting in loss of these cells and subsequent HPA axis dysfunction. This mouse model physiologically resembles a Pomc knockout mouse (Yaswen, et al. 1999) and highlights the importance of Notch signaling in the regulation of corticotrope ontogeny during HPA axis development. Our study indicates that subtle alterations in Notch signaling within POMC lineage cells during early embryonic development can lead to disruption of the HPA axis at the level of the hypothalamus, as well as the adrenal gland. Disruption of the HPA axis during development has significant physiological consequences on the body's response to stress, and can result in multiple disorders, including Cushing's syndrome and adrenal insufficiency (Drouin, et al. 2007). Our findings are not only important to the study of normal pituitary development, but also demonstrate how alterations in signaling within corticotropes can contribute to dysfunction along the entire HPA axis.

ACKNOWLEDGEMENTS

We are grateful to Drs. Jodi Flaws, Ann Nardulli and Phil Newmark for use of equipment, to Dr. Jeff Huang, Bonnie Zeigler, Jackye Peretz and Dr. David Forsthoefel for technical assistance and to Dr. Megan Mahoney for use of CRH antibody. The Pax7 antibody was kindly provided by the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Financial support was received from the National Institute of Health grant R01DK076647A (LTR) and T32 HD007333 (PKA).
REFERENCES


Nudi, M., Ouimette, J., Drouin, J., 2005. Bone Morphogenic Protein (Smad)-Mediated Repression of Proopiomelanocortin Transcription by Interference with Pitx/Tpit Activity. Molecular endocrinology (Baltimore, Md.) 5, 1329-42.


Figure 2.1. Persistent expression of NICD results in increases in mRNA levels of Notch targets. (A) Construct and mating scheme for activated NICD expression in *Pomc*-expressing cells. (B) Activated NICD results in modest increases in canonical target mRNA levels in Tg (gray bars) pituitaries as compared to control (black bars) at e16.5. n=3-5. *:p<0.05.
Figure 2.2. Persistent NICD expression inhibits the differentiation of melanotropes and corticotropes. Coronal sections of control (A) and Tg (B) e16.5 pituitaries were stained with hematoxylin and eosin. No differences in morphology were observed between the two groups. POMC immunoreactive cells (red) are found in the IL and AL of control (C) pituitaries at e16.5 and are diminished in both lobes of Tg (D) mice. Morphology is similar at p1 in control (E) and Tg (F) pituitaries. POMC expression (red) is observed in the AL and IL of control mice (G) and is markedly decreased in Tg pituitaries (H) at p1. RT-PCR at e16.5 reveals significant decreases in Pomc mRNA in Tg mice as compared to controls (I). Photos taken at 200x; Scale bars: 50μm. ****:p<0.0001. n=5-7 (immunohistochemistry) and n=3-5 (qRT-PCR).

PL=posterior lobe. AL=anterior lobe. IL=intermediate lobe.
Figure 2.3. Transcription factors regulating Pomc expression are decreased in pituitaries of NICD expressing mice at p1. *p<0.05. ***: p<0.001. n=3-5.
Figure 2.4. IL cells containing NICD express markers of pituitary progenitors. SOX2 (red) is expressed at p1 in the pituitary of control (A) and Tg (B) mice. In control animals, SOX2 immunoreactivity (red) is mostly observed lining the cleft of the pituitary (C, G), with nuclei stained with DAPI (blue; E, G). Although there are also scattered SOX2 immunoreactive cells in the IL, many cells are not SOX2 immunoreactive (compare E (nuclei stained with DAPI) and G). In contrast, nearly every cell in the IL of Tg mice is immunoreactive for SOX2 (D, compare F (nuclei stained with DAPI) and H). Expression of Pax7, a marker of intermediate progenitors was also examined. Pax7 immunoreactive cells (red) are observed in the IL of control (I) and Tg mice (J) at p1. Many cells lining the pituitary cleft at p1 do not express Pax7 in control mice (K, O with nuclei stained with DAPI (blue; M,O), arrows show Pax7-negative cell). Nearly every cell in the IL of Tg mice is immunoreactive for Pax7 (L, compare N (nuclei stained with DAPI) and P). Photos taken at 100x (A, B, I and J), 400x (C-H, K-P). White box (A and I) indicates where higher magnification photos were taken. Scale bars (A-P): 50μm. n=4-5.
Figure 2.5. Undifferentiated cells in Tg mice are incompatible with postnatal survival, resulting in adrenal dysfunction. Adolescent (p32) pituitaries were stained with hematoxylin and eosin to visualize morphology. Control (A) animals have a prominent IL, whereas Tg (B) animals have a very thin layer of IL cells (arrow) surrounding the PL. Nearly all of the IL lobe cells are immunoreactive for POMC (red) in the control (C) mice. In contrast, very few POMC-positive cells are observed in the IL of Tg mice (D). The same is true in the AL where POMC immunoreactive corticotropes are abundant in control mice (E) and nearly absent in Tg pituitaries (F). Similarly, qRT-PCR for *Pomc* reveals a significant decrease in *Pomc* mRNA in Tg adolescent pituitaries (gray bars) as compared to control littermates (black bars) in both males and females (G). *Pax7* mRNA, which is exclusive to the IL in the pituitary, is present at p5 in both experimental groups and decreases dramatically by p9 in Tg pituitaries, indicating IL cells are no longer present at this age in Tg animals. Adrenals (I) of Tg males and females are reduced in size as compared to control mice. Stress-induced corticosterone levels (J) are significantly decreased in male and female Tg mice (gray bars) when compared to control mice (black bars). Photos taken at 100x (A-D) and 400x (E & F). Scale bars: 50μm (A-F) and 1mm (I). **: p<0.01. ***: p<0.001. ****: p<0.0001. n=3-5 (RT-PCR), n=5 (immunohistochemistry) and n=4 (corticosterone assay). PL=posterior lobe, AL=anterior lobe, IL=intermediate lobe.
Figure 2.6. Corticotropin releasing hormone (CRH) expression in the paraventricular (PVN) hypothalamus of male mice at p32. CRH immunoreactivity appears lower in control mice (A) compared to Tg mice (B) in the medial parvocellular portion of the PVN (PaMP). Scale bar: 50μm. n=3.
Figure 2.7. Proliferation is unaffected by persistent NICD expression. Ki67 immunohistochemistry was performed on p1 control (A) and Tg pituitaries (B) and no difference in immunoreactivity (red) was observed. Similarly, no difference in Phopho-histone-H3 immunoreactivity (green) was observed between control (C) and Tg (D) pituitaries at p1. Nuclei are visualized with DAPI staining (blue; A-D). Additionally, qRT-PCR showed no change in Myc mRNA levels between control and Tg pituitaries at p1 (E). Photos taken at 200x. Scale bars: 50μm. n=4-5 (immunohistochemistry) and n=4 (RT-PCR).
Figure 2.8. A proposed model for the role of Notch signaling in pituitary cell specification. Notch signaling must be repressed for corticotrope, melanotrope and gonadotrope differentiation to occur. In addition to repressing differentiation, Notch signaling is also necessary for the activation of PROP1, which is required for the emergence of the PIT1 lineage. Hes1, another Notch effector molecule, is necessary for the specification of melanotropes.
CHAPTER 3: Notch signaling in postnatal pituitary expansion: proliferation, progenitors and cell specification

ABSTRACT

Mutations in Prop1 account for up to half of the cases of combined pituitary hormone deficiency that result from known causes. Despite this, few signaling molecules and pathways that influence Prop1 expression have been identified. Notch signaling has been linked to Prop1 expression, but the developmental periods during which Notch signaling influences Prop1 and overall pituitary development remain unclear. To test the requirement for Notch signaling in establishing the normal pituitary hormone milieu, we generated mice with early embryonic conditional loss of Notch2 (cKO) and examined the consequences of chemical Notch inhibition during early postnatal pituitary maturation. We show that loss of Notch2 has little influence on early embryonic pituitary proliferation, but is crucial for postnatal progenitor maintenance and proliferation. Additionally, we show that Notch signaling is necessary embryonically and postnatally for Prop1 expression and robust Pit1 lineage hormone cell expansion, as well as repression of the corticotrope lineage. Taken together, our studies identify temporal and cell-type specific roles for Notch signaling and highlight the importance of this pathway throughout pituitary development.

1 This chapter has been accepted in its entirety for publication in Molecular Endocrinology. Nantie, L.B., Himes, A.D., Getz, D. and Raetzman, L.T., 2014. Notch Signaling in the postnatal pituitary: proliferation, progenitors and cell specification. In press.
INTRODUCTION

Hypopituitarism, including combined pituitary hormone deficiency (CPHD), has a prevalence of 45.5 per 100,000 individuals (Regal, et al. 2001). Approximately 50% of known causes of CPHD result from mutations in the transcription factors PROP1 and PIT1 (Cogan, et al. 1998; Wu, et al. 1998; Fluck, et al. 1998; Kelberman, et al. 2009; Prince, et al. 2011). PROP1 is necessary for the transcription of PIT1, which is required for growth hormone (GH), thyroid stimulating hormone (TSHβ) and prolactin (PRL) expression. Therefore, patients with mutations in PROP1, have loss of these hormones. Although PROP1 is not necessary for gonadotrope or corticotrope cell differentiation, many patients also present with reductions in gonadotropin (LH and FSH) levels and acquired adrenocorticotrophic (ACTH) deficiency (Pernasetti, et al. 2000; Agarwal, et al. 2000; Bottner, et al. 2004). Mouse models of Prop1 deficiency have been fundamental to our understanding of the etiology of CPHD. Ames Dwarf (Prop1<sup>df</sup>) mice, which harbor a spontaneous point mutation in Prop1, and genetically engineered Prop1<sup>null</sup> mice both recapitulate the phenotype of humans with PROP1 mutations (Sornson, et al. 1996; Gage, et al. 1996a; Nasonkin, et al. 2004).

Although the importance of PROP1 in pituitary development and disease is well understood, few genes that regulate PROP1 expression have been elucidated. Identifying these factors is important, as mutations in upstream regulators may be responsible for additional genetic causes of CPHD. One pathway that has been implicated in the control of Prop1 is the Notch signaling pathway. In a mouse model of pituitary-specific loss of the essential Notch cofactor, Rbpj, a reduction in Prop1 transcript, detected by in situ hybridization, is observed. This correlates with reduced
expression of Pit1, Gh and Tshb. Furthermore, it was shown that RBPJ can bind to a canonical element within the first intron of the Prop1 gene, indicating the control of Prop1 by the Notch signaling pathway may be direct (Zhu, et al. 2006). Interestingly, Ames Dwarf mice have reduced levels of the receptor Notch2, although increased levels of the Dll1 ligand and target Hey1, suggesting that PROP1 may also regulate Notch signaling (Raetzman, et al. 2004; Mortensen, et al. 2011).

The Notch signaling pathway is an evolutionarily conserved pathway that is critical to the development of many organs, where its most characterized role is in progenitor maintenance. This pathway requires cell-to-cell contact between one cell containing the ligand and the other containing the Notch receptor. Activation of the cascade in the receptor-containing cell, results in cleavage of the intracellular domain (NICD) of the receptor. The NICD can then translocate into the nucleus and, in complex with Mastermind-like and RBPJ, drive the transcription of downstream targets. These targets are basic helix-loop-helix transcriptional repressors that inhibit the expression of pro-differentiation genes, thus maintaining the progenitor state of the cell (reviewed in (Kopan and Ilagan. 2009)). Notch2 and Notch3 are expressed in the embryonic pituitary and global loss of the Notch effector gene, Hes1, results in reduced proliferation, pituitary hypoplasia and loss of αMSH cells in the intermediate lobe (IL) (Raetzman, et al. 2004; Zhu, et al. 2006; Raetzman, et al. 2007; Monahan, et al. 2009). In contrast, overexpression of Notch receptors can inhibit the differentiation of gonadotropes, melanotropes and corticotropes (Raetzman, et al. 2006; Goldberg, et al. 2011). However, the contribution of individual Notch receptors during pituitary development, as well as the effects of pituitary-specific loss of these receptors, remains unclear.
In addition to its expression during embryonic development, Notch signaling components are present in the adult pituitary. Expression of these factors are restricted to the cells lining the pituitary cleft, which correlates with the localization of adult pituitary progenitors (Tando, et al. 2013). *In vitro* treatment of side population progenitor cells from the pituitary of adult mice or dissociated whole pituitary cells from adult rats with the Notch inhibitor DAPT, results in a decrease in the number and proliferation of progenitor cells. Conversely, *in vitro* treatment with a soluble Notch ligand, using the same experimental systems, results in increased progenitor cell number and proliferation (Chen, et al. 2006; Tando, et al. 2013). It is unclear whether similar effects would be observed *in vivo*, as cell-to-cell contact is an important aspect of the Notch signaling cascade.

Studies show that the pituitary undergoes a second wave of proliferation and cell differentiation during the first three weeks after birth in rodents, during which it expands greatly in size (Carbajo-Pérez and Watanabe. 1990). Some of this expansion is due to increases in cell volume, but progenitor and differentiated cells also proliferate during this period (Taniguchi, et al. 2000; Taniguchi, et al. 2001; Taniguchi, et al. 2002a; Taniguchi, et al. 2002b; Gaston-Massuet, et al. 2011). Additionally, the number of progenitor cells greatly decreases after postnatal day (p) 21 in mice, indicating these cells may play an important role in this phase of expansion (Gremeaux, et al. 2012). The constellation of Notch signaling components expressed and whether they are important for proliferation and differentiation during this time is unclear.

In order to further dissect the role of Notch signaling during these critical windows of development, we used both genetic and chemical inhibition of Notch signaling
throughout early development and specifically during postnatal gland expansion. In order to examine the contribution of Notch2 during embryonic pituitary development, we generated a mouse with conditional loss of Notch2 (cKO), beginning at embryonic day (e) 8.5 in Foxg1-expressing cells (Wang, et al. 2010). Loss of Notch2 does not appear to greatly affect overall embryonic pituitary development, but does result in decreased expression of Prop1. Rather, we show that Notch signaling components are highly expressed during the early postnatal period and both acute chemical Notch inhibition and Notch2 cKO mice display decreases in pituitary proliferation and alterations in hormone cell proportions. These changes parallel a substantial decrease in Prop1 expression. Taken together, these studies further solidify Prop1 as a bona fide Notch target and identify a critical role for Notch signaling in postnatal pituitary development. Further studies will be necessary to identify whether altered Notch signaling could be a contributing factor in CPHD.

MATERIALS AND METHODS

Mice

For Notch signaling pathway component analysis, wildtype mice of mixed genetic background were used. For in vitro and in vivo DAPT treatment studies, CD-1 mice (Charles River, Wilimington, MA), from a breeding colony maintained in our laboratory, were used. For all studies, p1 is considered to be the day of birth. All animals were housed in a facility with a 12 hour light-dark cycle and were maintained in accordance with the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee.
In order to generate Notch2 cKO mice, Notch2^fl/fl mice (McCright, et al. 2006) were obtained from Jackson Laboratory (Bar Harbor, ME) and bred to Foxg1^+/cre mice, (Hebert and McConnell. 2000) maintained on a 129 background, also purchased from Jackson Laboratory. The resulting Notch2^+/fl, Foxg1^+/cre mice were bred to Notch2^+/fl, Foxg^+/+ littermates to obtain cKO animals. To genotype the mice, tail biopsies were obtained and DNA was extracted using a hot shot or salt-out method. PCR for the Cre and Notch2 alleles was performed as previously described (Himes and Raetzman. 2009) using published primer sequences (Hebert and McConnell. 2000; McCright, et al. 2006).

**DAPT injections**

Beginning at p2, CD1 mice were injected daily for 3 days with 100mg/kg DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester, Gamma Secretase Inhibitor IX, Millipore, Billerica, MA) diluted in Dimethyl Sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) or an equivalent volume of DMSO alone. Pituitaries and whole heads were harvested 24h after last injection. For studies examining hormone cell fate choice, mice were injected with 100mg/kg DAPT or an equivalent volume of DMSO for five days beginning on p3. Pituitaries were harvested on p12, five days after last injection. For bromodeoxyuridine (BrdU) studies, mice were injected subcutaneously with 0.1mg/g body weight BrdU (Sigma-Aldrich, St. Louis, MO), diluted in water, 30 minutes prior to harvest.

**Pituitary explant culture and DAPT washout assay**

Pituitaries were dissected from p3 CD1 mice, rinsed with PBS and transferred to a 96 well plate. Culture media was made by adding 1% Penicillin Streptomycin (Fisher,
Fairlawn, NJ) and 10% fetal bovine serum (HyClone, Logan, UT) to DMEM/F-12 media (Cellgro, Manassas, VA). 10µM DAPT dissolved in DMSO (Millipore) or an equivalent amount of DMSO (Cambridge Isotope Laboratories, Andover, MA) was added to the culture media from the beginning of culture. After 24h in culture, media was spiked with DAPT or DMSO (an equivalent amount to what was added at the beginning of culture) and pituitaries were cultured for an additional 24h. For washout assays, media was removed from pituitaries, pituitaries were rinsed and DAPT-free, DMSO-containing media or 10µM DAPT-containing media was added. Pituitaries were harvested 6h later.

**Immunohistochemistry**

Whole e12.5 embryos, heads of postnatal mice and pituitaries were fixed in 3.7% formaldehyde (Sigma Aldrich, St. Louis, MO) diluted in phosphate buffered saline (PBS) and embedded in paraffin for sectioning. Slides were deparaffinized and rehydrated. For immunohistochemical detection of SOX2, SOX9, Ki67, PH3, BrdU and Pit1 samples were boiled in 10 mM citrate solution, pH6 for 10 minutes and cooled for 15 minutes. Next, all samples were blocked in a solution containing 5% normal donkey serum (Jackson Immunoresearch, West Grove, PA) diluted in immunohistochemical block, which contains 3% bovine serum albumin (Jackson Immunoresearch) and 0.5% TritonX-100 (Sigma Aldrich, St. Louis, MO) diluted in PBS. Primary antibodies used were raised against the following peptides: SOX2 (1:2000, Millipore, Billerica, MA), phospho-histone-H3 (PH3, 1:500, Upstate Cell Signaling Solutions, Lake Placid, NY), Ki67 (1:500 BD Biosciences, San Jose, CA) SOX9 (1:500, Millipore, Billerica, MA), Pit1 (1:4000, kind gift of Dr. Simon Rhodes), POMC (1:1000, DAKO, Carpinteriam, CA), GH, TSHβ and LHβ (1:1000, Dr. A.F. Parlow and the National Hormone and Pituitary
Program, University of California, Los Angeles) and BrdU (1:200, AbD Serotec, Raleigh, NC). Slides were then incubated with biotin-conjugated rat (BrdU), mouse (Ki67) or rabbit (Pit1, GH, TSHβ, POMC, LHβ, SOX2, SOX9 and PH3) secondary antibody. This was followed by incubation in a streptavidin-conjugated cy3 fluorophore. Biotin and streptavidin-conjugated antibodies were purchased from Jackson ImmunoResearch and were used at a concentration of 1:500. Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:1000, Life Technologies, Grand Island, NY) and visualized at 50x, 100x, 200x, or 400x magnification using a Leica DM2560 microscope. Photographs were taken using a Retiga 2000R color camera (Q-Imaging, Surrey, Canada) and acquired using Q-Capture Pro software (Q-Imaging). Images were processed using Adobe Photoshop CS4 (San José, CA).

Pit1, POMC, LHβ BrdU quantification

In order to quantify cells incorporating BrdU, three equally spaced slides containing three sections each, were immunohistochemically stained for BrdU. For Pit1, POMC and LHβ cell number quantification, 5-6 equally spaced slides containing two sections each, were immunohistochemically stained using their respective antibodies. For BrdU, the number of positive cells in the AL and IL were counted and the area of the AL and IL was measured. For Pit1, POMC and LHβ quantification, the number of positive cells in the AL was counted and the area of the AL was measured. Additionally, for all cell counts, a ratio of cells per area was determined for each group by counting the number of DAPI cells per area. This ratio was used to calculate the total cells in the each pituitary section. The number of BrdU-, Pit1-, POMC- or LHβ-positive cells was divided by the calculated total cells in each pituitary, giving the percent of
immunopositive cells. Statistical significance was determined using Student’s $t$ test.

**AL Volumetric Approximation**

For volumetric approximation, every third slide, containing two sections each, of p6 control and cKO mice was stained with DAPI. The area of the AL was measured for each stained section using ImageJ (NIH, Bethesda, MD). In order to obtain an approximation of the AL volume, this area was multiplied by the thickness of the section and by three, to account for slides in between stained slides.

**In Situ Hybridization**

Slides were deparaffinized, rehydrated and incubated in PBS containing 0.3% triton-X 100 (Sigma-Aldrich, St. Louis, MO) for 10 minutes and permeabilized using proteinase K (Life Technologies, Foster City, CA) for fifteen minutes at 37°C. Samples were then post-fixed using 3.7% formaldehyde (Sigma-Aldrich) and acetylated for ten minutes in a solution containing 0.25% acetic anhydride (Sigma-Aldrich) diluted in triethanolamine (Sigma-Aldrich) buffer. Following acetylation, sections were incubated in hybridization solution at 55-58°C for a minimum of two hours. DNA Digoxigenin-UTP (DIG) labeled probes for the *Prop1* (Cushman, et al. 2001), *Notch2* (kind gift of Dr. Shelley Ross, Raetzman, et al. 2004), *Hes1* (kind gift of Dr. Ryoichiro Kageyama), *Hey1* (kind gift of Dr. Mannfred Gessler) or *Ghrh* (kind gift of Drs. Iain Robinson and Paul LeTissier) were denatured and applied to slides for overnight incubation at the hybridization temperature. Next, samples were treated with a solution of 50% formamide (Sigma-Aldrich) for one hour at the hybridization temperature, followed by blocking and then application of anti-DIG Fab fragments for one to two hours at room temperature. Slides were then washed, treated with Chromagen buffer solutions of
increasing pH and developed at room temperature overnight in Chromagen buffer containing NBT/BCIP. Dig-labeled nucleotides, anti-dig antibody and NBT/BCIP were purchased from Roche (Mannheim, Germany).

Quantitative Reverse Transcriptase PCR

RNA was isolated from embryonic and postnatal whole pituitaries using an RNAqueous micro kit (Ambion, Austin, TX) as per manufacturers protocol. For p21 and adult pituitaries, 0.5µg of RNA was synthesized into cDNA using the ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA). For e16.5 and p1, p5 and p10 and p12 pituitaries, the total amount of RNA from each pituitary was converted into cDNA. A no Reverse Transcriptase enzyme control was also prepared and used as a negative control. For quantitative RT-PCR 0.1-0.33 µL of cDNA was amplified using gene-specific primers and SYBR green mix (Bio-Rad Laboratories, Hercules, CA) on a Bio-Rad MyIQ real-time PCR machine. Data were analyzed with the change in cycle threshold (ΔCt) value method (Goldberg, et al. 2011). Statistical significance was determined using Student’s t test. For a list of gene specific primers, see Table 3.1.

RESULTS

Loss of Notch2 does not affect proliferation or corticotrope differentiation at e12.5.

At e12.5, the morphology of the developing pituitary appears similar between control (Figure 3.1A) and Notch2 cKO mice (Figure 3.1B). Additionally, proliferation appears to be largely unchanged between control (Figure 3.1C) and cKO (Figure 3.1D) pituitaries. POMC immunostaining reveals few positives cells in control (Figure 3.1E)
and cKO mice (Figure 3.1F), indicating there is no apparent alteration in corticotrope number when Notch2 is lost, contrary to what is observed in the Prop1, Hes1 double knockout (Himes and Raetzman. 2009). Because no striking phenotype is observed in Notch2 cKO mice, we performed in situ hybridization for Hey1 in control and cKO mice to determine if a canonical Notch signaling target is reduced as expected. In control mice, at e12.5, Hey1 is expressed throughout the developing pituitary (Figure 3.1G). In cKO mice, there is a marked reduction, although not complete loss, of Hey1 expression throughout the developing pituitary (Figure 3.1H). Because Notch signaling has been shown to potentially regulate Prop1, we examined Prop1 expression in control and cKO developing pituitaries. In control mice, Prop1 is expressed throughout Rathke’s pouch (RP) and is restricted away from the ventral portion of the developing pituitary where differentiated cells are housed (Figure 3.1I). In cKO mice, Prop1 appears to be reduced throughout RP (Figure 3.1J). Taken together, these data suggest that Notch2 is not necessary for proliferation during early development, but is necessary for Prop1 expression.

Characterization of Notch signaling components during postnatal expansion phase of pituitary development

The pituitary undergoes two distinct waves of cell proliferation and differentiation, one occurring during embryogenesis and a second that takes place during the first three postnatal weeks in the mouse (Zhu, et al. 2007; Gremeaux, et al. 2012). Although it appears Notch2 is not necessary for early pituitary development to proceed normally, the role of Notch signaling during the second wave of proliferation and differentiation has yet to be elucidated. In order to address this question, we first investigated the
spatial and temporal expression patterns of Notch signaling components during this critical window to determine if this pathway is active during this time. We used qRT-PCR to determine the relative levels of Notch family members during this time (e16.5, p1, p5, p10, p21) and compared these levels to those observed in the adult pituitary (Figure 3.2A-3.2C). These data show that Notch signaling components are present during this wave of pituitary expansion and their mRNA levels are, in general, at higher levels than those found in adult pituitaries.

In order to determine the mRNA localization of Notch targets and receptors, we performed in situ hybridization for Notch2, Hey1 and Hes1 in p5 coronal sections. We find that Notch2 mRNA is mainly restricted to some, but not all cells surrounding the pituitary cleft, particularly in the area where the intermediate and anterior lobes intersect. There are also Notch2-positive cells scattered throughout the anterior lobe (AL, Figures 3.3A and 3.3B). This expression pattern correlates with the localization of the population of pituitary progenitors containing SOX2 (Fauquier, et al. 2008). A similar expression pattern is observed for Hey1 (Figures 3.3C and 3.3D), with positive cells seen lining the cleft and in scattered cells in the AL. Hes1 appears to be in fewer cells than Notch2 and is restricted to the AL side of the pituitary cleft (Figures 3.3E and 3.2F). Recent studies have shown that PROP1 is also present in cleft cells after birth (Garcia-Lavandeira, et al. 2009; Tando, et al. 2013).

These findings indicate that Notch signaling components are present during early postnatal pituitary development and are dynamic, warranting further investigation of the role of this signaling pathway during this period. We examined the expression of Notch receptors in Notch2 cKO mice at p1 and find that Notch2 is specifically reduced,
although not completely lost (Figure 3.3G). Additionally, we find significant reductions in mRNA levels of Notch targets, especially Prop1 (Figure 3.3H), making the cKO mouse a good model to investigate the effects of loss of Notch2 at this age.

Notch2 is necessary for the maintenance of pituitary progenitors after birth

Our analysis shows that Notch2 is maintained in cleft cells after birth, indicating it may have a role in progenitor maintenance during the postnatal wave of pituitary development. We, therefore, examined SOX2 expression at p1 in control and cKO mice. At p1, SOX2 is normally expressed in most cells lining the cleft of the pituitary on both the AL and IL sides. In addition, SOX2 is also found in cells scattered throughout the IL and the AL. (Figure 3.4A, 3.4C and 3.4E). In cKO mice, none of the cells lining the pituitary cleft on the IL side are positive for SOX2. Instead, SOX2-immunoreactive cells are found in the dorsal IL, at the IL-posterior lobe (PL) border (Figure 3.4B and 3.4D). Interestingly, cells in the AL appear to be largely unaffected in localization or number in the cKO (Figure 3.4B and 3.4F). The expression pattern of SOX9, another progenitor marker, is similar to that of SOX2. In control mice at p1, SOX9-positive cells are found lining the pituitary cleft and scattered throughout the IL and AL, as well as in many cells in the PL (Figures 3.4G, 3.4I and 3.4K). In cKO mice, SOX9 is not found in cells lining the cleft in the IL, but, similar to SOX2 localization, is restricted to cells in the dorsal IL (Figures 3.4H and 3.4J). In addition, in cKO mice, the number of SOX9-immunoreactive cells lining the cleft and in the parenchyma of the AL appears to be reduced (Figures 3.4H and 3.4L, arrows indicate SOX9-negative cleft cells). In support of these observations, mRNA levels of Sox2 and Sox9 are both significantly decreased in cKO
mice as compared to controls (Figure 3.4M). Taken together, these results show that
Notch2 is necessary for the maintenance and localization of progenitor cells at p1.

Loss of Notch2 results in a progressive decrease in pituitary progenitor number

To assess whether progenitor cells are maintained in the Notch2 cKO throughout
early postnatal expansion, we stained p5 control and cKO pituitaries for SOX2 and
SOX9. In control mice, SOX2 expression in the IL is mostly limited to cells lining the
cleft (Figure 3.4N and 3.4P). In cKO mice, fewer SOX2-expressing cells remain in the IL
than what is observed at p1. The cells that do remain are still present at the IL-PL
border (Figure 3.4O and 3.4Q), rather than lining the cleft. On the AL side of the cleft,
SOX2 is present in nearly every cell in control mice (Figure 3.4N and 3.4R), and
appears to be expressed in fewer cells in cKO mice (Figure 3.4O and 3.4S, arrows). In
addition to SOX2-positive progenitors lining the cleft, there are clusters of SOX2-
expressing cells in the AL parenchyma (Figure 3.4T). Although these clusters are still
present in cKO mice, they appear to be reduced (Figure 3.4U). In control mice, SOX9
expression follows a similar pattern in the IL to that of SOX2 (Figure 3.4V and 3.4X).
Likewise, SOX9 is only expressed in few cells in the IL of cKO and these cells are
present only in the dorsal IL (Figure 3.4W and 3.4Y). As with SOX2, SOX9 is expressed
in nearly every cell lining the cleft in the AL in control mice (Figure 3.4V and 3.4Z).
However, SOX9 expression appears to be lost in many AL cleft cells in cKO mice
(Figure 3.4W and 3.4A′, arrows). SOX9 is expressed in clusters in the AL parenchyma
in control mice (Figure 3.4B′). In contrast, very few, if any, clusters are observed in the
AL parenchyma of cKO mice, and the SOX9-positive cells that are present appear to be
morphologically different than their control counterparts (Figure 3.4C′). Taken together,
these data suggest that Notch2 is necessary to maintain and expand the progenitor
population during postnatal pituitary maturation.

Loss of Notch2 results in decreased pituitary proliferation at p1 and decreased AL
volume at p6

Although proliferation is not affected at e12.5 when Notch signaling is reduced,
we observe a disruption of progenitor cells at p1 and p5. Therefore, we examined
whether these changes in progenitor cell number and localization correlate with
changes in postnatal proliferation. In control mice, Ki67-immunoreactive cells are
observed throughout the AL, IL and PL (Figure 3.5A). In the IL, Ki67 expression is found
throughout, especially in cells lining the cleft (Figure 3.5C). In cKO mice, there appears
to be an overall reduction in the number of Ki67-expressing cells in the AL and a striking
reduction of immunopositive cells in the IL (Figures 3.5B and 3.5D). Additionally, an
apparent decrease in cells in mitosis is also observed in the cKO as evidenced by a
reduction in the number of cells expressing phospho-histone H3 (PH3, Figure 3.5F) as
compared to control (Figure 3.5E). Furthermore, mKi67 and Ccne1 mRNA levels are
reduced in cKO pituitaries. This is coincident with an increase in Cdknc1 (p57) mRNA
(Figure 3.5G), a cell cycle inhibitor shown to be necessary for pituitary progenitors to
exit the cell cycle before differentiating (Bilodeau, et al. 2009). Together, these data
indicate that Notch2 is an important regulator of pituitary proliferation at p1.

Because we observe decreased proliferation at p1, we examined approximate AL
volume at p6 to determine how these changes in proliferation may result in changes in
pituitary size. We find that at p6 the AL of cKO mice (Figures 3.5J and 3.5K) appears
hypoplastic compared to control (Figures 3.5H and 3.5I). Additionally, the approximate
volume of the cKO AL is significantly reduced as compared to control (Figure 3.5L). These findings suggest that reduction in Notch2 can lead to a hypoplastic pituitary as early as p6.

**Chemical Notch inhibition results in decreased postnatal proliferation in vivo**

In Notch2 cKO mice, we do not observe proliferation defects until after birth, indicating Notch signaling may be specifically important for proliferation that occurs postnatally. In order to confirm the postnatal changes we observe in Notch2 ckO mice are due to direct actions postnatally, we injected mice during early postnatal development with the gamma secretase inhibitor DAPT \((N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester)\). DAPT inhibits cleavage of the Notch intracellular domain, thus preventing activation of the downstream pathway (Dovey, et al. 2001; Sastre, et al. 2001; Geling, et al. 2002). Mice were injected with 100 mg/kg DAPT or vehicle control (DMSO) daily between p2 and p4 and pituitaries were harvested 24 hours after last injection (Figure 3.6A). This time period was chosen because, based on expression analysis, many Notch signaling components appear to be most highly expressed between p1 and p5. To determine the efficacy of Notch inhibition in DAPT-injected mice, mRNA levels of the canonical Notch targets *Hes1* and *Hey1* and the putative pituitary-specific Notch target *Prop1* were examined in whole pituitaries. We see a significant decrease in all targets examined, with *Prop1* levels being most highly suppressed after DAPT treatment as compared to vehicle-treated controls (Figure 3.6B).

Because Notch signaling is known to be important for proliferation in many developing organs and because we observe decreases in proliferation at p1 in Notch2
cKO mice, we examined proliferation by quantifying the percent of BrdU positive cells in
the AL and IL of pituitaries of DAPT-treated mice and compared these values to vehicle
controls. In vehicle-injected mice there are an average of 5.51% (+/- 0.20%) of cells
that are positive for BrdU (Figure 3.6C) as compared to 2.98% (+/- 0.26%) of cells in
pituitaries of DAPT-treated mice (Figure 3.6D). Additionally, we find a reduction in
mRNA levels of mKi67, which is present in cells during all phases of the cell cycle, and
in Ccnd1, which is important for transition from G1 to S phase, in mice treated with
DAPT (Figure 3.6E). This is coincident with an increase in Cdknc1 (p57) mRNA (Figure
3.6F). These observations show that Notch signaling contributes to postnatal pituitary
proliferation in vivo and are similar to those seen in Notch2 cKO mice at p1.

In vitro chemical Notch inhibition reveals direct effects on the pituitary

To elucidate whether the effects observed in the in vivo DAPT injection study are
consistent with direct effects on the pituitary, we employed an in vitro organ culture
system (Figure 3.6G). We examined whether proliferation in DAPT-cultured pituitaries is
affected in an analogous manner to that seen in DAPT-injected mice. Similarly, we find
a significant decrease in mRNA levels of mKi67 in pituitaries treated with DAPT as
compared to vehicle treated pituitaries after 48h in culture (Figure 3.6H). Taken
together, the Notch2 cKO and acute DAPT treatment studies implicate Notch signaling
in maintaining at least a subset of progenitors and allowing their proliferation during
postnatal development.

To identify potentially direct targets of Notch signaling, we performed a 6h
washout assay, using the in vitro explant system. Targets that are altered after DAPT
treatment, but recover within the 6h period, are considered to be likely direct targets of
the Notch signaling pathway (Weng, et al. 2006; Chadwick, et al. 2009; Wang, et al. 2011). We observe significant reductions in mRNA levels of the Notch targets Hes1, Hey1 and Prop1, with Prop1 levels again being the most decreased after culture in the presence of DAPT. Six hours after washout, the mRNA levels of the canonical Notch targets Hes1 and Hey1 have recovered to levels comparable to those in vehicle-treated pituitaries. Prop1 levels recover to levels that are statistically similar to vehicle-treated pituitaries, albeit not as complete of a response as Hes1 or Hey1 (Figure 3.6I). This further suggests that Prop1 is likely a direct Notch target and show that Notch signaling can influence Prop1 expression after birth.

Loss of Notch2 results in decreased Pit1 cell number and increased POMC number at p1

In addition to its role in progenitor maintenance and proliferation, Notch signaling has been shown to be a critical regulator of cell fate in many systems (Ohtsuka, et al. 2001; Murtaugh, et al. 2003; Patten, et al. 2003; Siveke, et al. 2008). In order to determine whether Notch signaling has a similar role in the pituitary, we examined the fate of Pit1-lineage cells, which contain GH, TSH and PRL, as well as corticotropes and gonadotropes at p1 in control and Notch2 cKO mice. GH is normally expressed in the AL of control mice (Figure 3.7A) and appears to be reduced in cKO pituitaries (Figure 3.7B). TSHβ appears throughout the AL in control mice (Figure 3.7C) and appears to be largely unchanged in cKO pituitaries (Figure 3.7D). In parallel, we examined mRNA levels of Gh, Tshb and Pou1f11 (Pit1). We observe a significant reduction in cKO mRNA levels of Pou1f1 and Gh, with Tshb levels statistically similar between control and cKO mice (Figure 3.7Q). To determine if the observed changes in mRNA levels
correlates with changes in cell number, we quantified the % of Pit1-positive cells in the AL of control and cKO mice. In control mice 44.35% (+/- 1.59%) of AL cells are positive for Pit1, compared with only 26.97% (+/- 2.85%) in cKO mice (Figure 3.7T), suggesting Notch2 is, at least in part, necessary to obtain the proper number of Pit1-positive cells.

POMC is expressed in melanotropes in the IL and corticotropes in the AL in both control (Figure 3.7E) and cKO (Figure 3.7F) mice and appears to be slightly increased in the AL of cKO mice as compared to control. Additionally, we observe a trend towards an increase in mRNA levels of Tpit and Pomc, which are present in both corticotropes and melanotropes, in cKO mice. To more specifically explore corticotrope and melanotrope lineages, we examined expression levels of genes that are specific to each cell type (Budry, et al. 2012). We show an increase in levels Crhr1, which is expressed in corticotropes, in cKO pituitaries. Conversely, no changes in mRNA levels of Pax7, a melanotrope-specific marker, are observed in cKO mice (Figure 3.7R). In order to determine if these changes in mRNA are coincident with changes in cell number, we quantified the number of POMC-positive cells in the AL. Control mice have 11.87% (+/- 0.40%) POMC-expressing cells in the AL. cKO mice have a significantly increased number of POMC-containing AL cells (15.49% +/- 0.83%) (Figure 3.7T), suggesting that Notch2 is an important factor regulating corticotrope number.

LHβ is found in few cells scattered throughout the ventral pituitary in control mice (Figure 3.7G) and the number of LHβ-positive cells does not appear to be altered in cKO pituitaries (Figure 3.7H). Although the number of LHβ-immunopositive cells does not appear to be changed in cKO mice, Lhb mRNA levels are significantly increased in cKO pituitaries as compared to control. Additionally, Nr5a1, a transcription factor
expressed only in gonadotropes in the pituitary is significantly increased in cKO at p1, while \textit{Gnrhr} shows a trend towards an increase (Figure 3.7S). To determine if these changes in mRNA levels correlate with changes in LHβ-positive cell number, we quantified the percentage of AL cells that express this hormone. We find no difference between control (2.95% +/- 0.20%) and cKO mice (2.99% +/- 0.08%) (Figure 3.7T), suggesting regulation of the gonadotrope cell number is likely independent of \textit{Notch2}. Mice with conditional loss of \textit{Notch2} have reduced GH and TSHβ at p6.

In order to examine how loss of \textit{Notch2} affects expansion of the pituitary hormone lineages during postnatal development, we immunohistochemically examined the expression of pituitary hormones at p6. Similar to what was observed at p1, GH appears to be reduced in cKO pituitaries (Figure 3.7J) as compared to control pituitaries (Figure 3.7I). In contrast to p1, TSHβ appears to be strongly reduced in cKO mice at p6 (Figure 3.7L) as compared to control mice (Figure 3.7K). POMC is present in the IL and AL of control mice (Figure 6M) and appears to be slightly increased in the AL of cKO mice (Figure 3.7N), mirroring the qRT-PCR results and cell counts observed at p1. LHβ is present in scattered AL cells of control mice (Figure 3.7O) and cKO mice (Figure 3.7P) and appears similar between the two groups. Taken together, these data show that \textit{Notch2} is necessary to control the number of Pit1-positive cells and POMC-positive cells during postnatal pituitary expansion.

In order to eliminate the possibility that the reduction in \textit{Gh} is a result of the presence of the Cre transgene alone, we examined \textit{Gh} mRNA in control mice with (\textit{Notch2}^{+/+} Foxg1^{+/cre} or \textit{Notch2}^{+/fli} Foxg1^{+/cre}) and without (\textit{Notch2}^{+/+} Foxg1^{+/+}, \textit{Notch2}^{+/fli} Foxg1^{+/+} or \textit{Notch2}^{fli/fli} Foxg1^{+/+}) the Cre transgene. No differences were observed
between the two groups in $Gh$ levels (Figure 3.8A) at p1. Additionally, although the $Foxg1$ Cre should not be expressed in the developing hypothalamus and thus $Notch2$ expression should not be altered there, we examined whether reduced GH expression could be due to unexpected alterations in $Ghrh$ in the hypothalamus in the cKO mice. In control mice, $in situ$ hybridization reveals $Ghrh$ mRNA is present in cells lateral to the third ventricle in the arcuate nucleus (Figure 3.8B). A similar pattern of expression is observed for cKO mice (Figure 3.8C). These data suggest that the phenotype of the $Notch2$ cKO mice is likely due to the pituitary intrinsic effects of loss of $Notch2$.

Postnatal chemical Notch inhibition results in decreased mRNA levels of Pit1-lineage hormones and increased $Pomc$ mRNA levels

Because alterations in pituitary hormones were observed when $Notch2$ is lost from early embryogenesis, we investigated whether Notch signaling is also specifically important for postnatal pituitary cell fate choice. In order to examine this question, we injected mice with DAPT daily from p3 through p7 and harvested whole pituitaries on p12 in order to allow changes in differentiated cells to manifest (Figure 3.9A). In mice treated with DAPT, we observe a significant decrease in mRNA levels of $Pou1f1$, $Prl$ and $Tshb$. Interestingly, although part of the Pit1 lineage, mRNA levels of $Gh$ are unchanged after DAPT treatment. $Pomc$ mRNA levels are increased after DAPT treatment, while $Lhb$ mRNA levels appear unaffected (Figure 3.9B). In order to determine whether these alterations are maintained after DAPT treatment, we injected mice with DAPT daily from p3 through p7 and harvested their pituitaries on p32 (Figure 3.9C). We show that mRNA levels of $Pou1f1$ and $Prl$ remain decreased in DAPT-treated mice, whereas all other hormones are unchanged (Figure 3.9D). These findings show
that Notch signaling is necessary for proper mRNA levels of *Pou1f1, Tshb, Prl*, and *Pomc* and suggest a role for Notch in postnatal pituitary cell fate choice, similar to what was uncovered in the *Notch2* cKO mouse.

**DISCUSSION**

Taken together, our studies have defined key roles for Notch signaling during postnatal development. We show that Notch signaling is required for pituitary proliferation and progenitor maintenance during postnatal pituitary expansion, but appears to be less important for these processes during embryonic development. Furthermore, during both periods of development, we show that Notch signaling regulates the balance between the Pit1 and POMC lineages.

We have identified *Notch2* as an important regulator of postnatal pituitary progenitor placement and number. Pituitary stem/progenitor cells contain the markers SOX2 and SOX9 and have been shown to contribute to postnatal pituitary homeostasis during both normal and stressed conditions (Rizzoti, et al. 2013; Andoniadou, et al. 2013). Therefore, it is critical that an appropriate number of progenitor cells are maintained. We show that *Notch2* cKO mice have progressive loss of this progenitor population after birth, suggesting that Notch signaling is necessary for maintenance of these cells. Loss of SOX2 in the embryonic pituitary results in impaired proliferation, pituitary hypoplasia and a reduction in cells of the Pit1 lineage (Jayakody, et al. 2012), mirroring what is observed in *Notch2* cKO mice. Additionally, overexpression of activated Notch in POMC-expressing cells results in increased SOX2 expression in the IL (Goldberg, et al. 2011), suggesting Notch may have the ability to induce SOX2
expression. Both Sox2 and Sox9 are direct Notch targets in neural progenitors \textit{in vivo} (Li, et al. 2012), indicating the potential for Notch to directly control these factors in the pituitary.

Loss of \textit{Notch2} results in differential effects in AL and IL progenitors. IL progenitors are misplaced at p1, similar to what is observed with conditional loss of the Notch pathway components \textit{Numb} and \textit{Numblike} (Moran, et al. 2011) and are nearly absent by p5. This is likely due to the fact that they lack the proper progenitor niche. In contrast, AL progenitors appear to be correctly localized but decreased in number by p5. These differences may be due to the heterogeneity of progenitor populations observed throughout the pituitary (Garcia-Lavandeira, et al. 2009; Rizzoti. 2010). Additionally, we show that \textit{Notch2} is not uniformly expressed in all progenitors, which may contribute to differences observed in different populations. These differential effects in progenitor populations may also be a contributing factor to the changes in hormone producing cell number observed at p1, as some lineage-committed or intermediate progenitors may have altered ability to expand.

We show that reductions in \textit{Notch2} have little effect on embryonic pituitary proliferation, but that \textit{Notch2} is required for proliferation during postnatal pituitary expansion. This is in contrast to studies showing reduced proliferation in the embryonic pituitary of \textit{Hes1} knockout mice (Zhu, et al. 2006; Raetzman, et al. 2007; Kita, et al. 2007; Monahan, et al. 2009). This indicates that the \textit{Hes1} null phenotype may be due to factors extrinsic to the pituitary or that the reduced but detectable presence of \textit{Hes1} and other Notch signaling components, including \textit{Notch3}, in \textit{Notch2} cKO mice is sufficient to maintain embryonic proliferation. Additional studies examining pituitary-specific loss of
*Hes1* will be required to further explore this question. Although we do not observe alterations in proliferation in cKO mice at e12.5, we see a striking decrease in proliferation at p1. We see a similar reduction in proliferation in mice treated postnatally with the chemical Notch inhibitor DAPT. This correlates with studies showing decreased proliferation in adult dissociated pituitary cells *in vitro* when treated with DAPT (Chen, et al. 2006; Tando, et al. 2013). However, it remains unclear if Notch signaling directly controls proliferation or alters the ability of stem cells to respond to proliferative signals.

We show that loss of *Notch2* results in alterations in cell fate, a phenomenon that has been described in multiple contexts, including pancreatic and central nervous system development (Ohtsuka, et al. 2001; Murtaugh, et al. 2003; Patten, et al. 2003; Siveke, et al. 2008). Specifically, after loss of *Notch2*, we detect a decrease in Pit1 cell number. This finding is similar to that observed in a conditional pituitary knockout of *Rbpj*, an essential Notch cofactor (Zhu, et al. 2006). Additionally, we have extended these observations showing that alterations in hormone producing cell number are maintained during postnatal expansion in *Notch2* cKO mice and that the Pit1 lineage is similarly affected after postnatal DAPT treatment. It is likely that the changes observed in the Pit1 lineage in both models are due to reductions in *Prop1* levels as *Prop1* is necessary for differentiation of cells of the Pit1 lineage (add reference here). However, it is also likely that the levels of *Prop1* in *Notch2* cKO and DAPT-injected mice are sufficient to allow for some expression of Pit1, as we do not observe a complete absence of this lineage. Taken together, our results show that reduced levels of *Notch2*, or of Notch signaling components in general, result in specific and incomplete reduction in Pit1 and hormones of the Pit1 lineage. This suggests that other signaling pathways or
molecules are also important for development and function of Pit1 lineage hormones. Such factors may include GATA2, which helps to maintain Tshb expression (Kashiwabara, et al. 2009) and Math3, which is required for somatotrope maturation (Zhu, et al. 2006).

In addition to the decrease observed in Pit1 lineage cells when Notch is reduced, we find that the POMC lineage is also affected by reductions in Notch signaling. In Notch2 cKO mice, we observe an increase in the percent of POMC-containing AL cells and an increase in mRNA levels of Crhr1, which is restricted to corticotropes, in Notch2 cKO mice. These changes are paralleled by an increase in Pomc mRNA in mice treated with the chemical Notch inhibitor, DAPT. A similar increase in POMC-expressing cells is observed in mice with loss of both Hes1 and Prop1 and in mice with pituitary-specific loss of Rbpj (Zhu, et al. 2006; Himes and Raetzman. 2009). Additionally, in order to obtain ACTH-producing cells from embryonic stem cells, these cells must first be incubated with DAPT (Suga, et al. 2011). This is confirmed by the observation that persistent expression of activated Notch in corticotropes results in POMC repression (Goldberg, et al. 2011). Taken together, these data all suggest that Notch signaling must be inhibited for corticotrope differentiation to proceed. Interestingly, the IL POMC-expressing melanotropes appear to be less affected by alterations in Notch signaling in our model. This may be due to the fact that we observe no changes in Pax7, a pioneer factor that dictates melanotrope fate by altering chromatin accessibility (Budry, et al. 2012). Additionally, despite these changes in corticotrope and Pit1 lineage cell number, our data suggest the gonadotrope lineage differentiates independently of Notch signaling. Taken together, these data strongly suggest that Notch2 may help to regulate
the balance between the Pit1 and POMC lineages. This role may be direct or may be a result of the altered ability of different progenitor populations to expand.

Our studies have defined Notch2 as a critical regulator of Prop1 expression. We show that loss of Notch2 leads to decreased expression of Prop1 at both e12.5 and p1 and that in vivo and in vitro exposure to the chemical Notch inhibitor DAPT results in strong reductions in mRNA levels of Prop1. A recovery in Prop1 mRNA levels in vitro is observed six hours after DAPT washout, mirroring the recovery seen in the canonical Notch targets Hes1 and Hey1. Recent work has shown that RBPJ can bind to the first intron of the Prop1 gene, further solidifying Prop1 as a direct Notch target (Zhu, et al. 2006). Additionally, the phenotype observed in Notch2 cKO mice is similar, although less severe, to that observed in Prop1-deficient mice, with both showing a reduction in hormones of the Pit1-lineage and inability of the pituitary to appropriately expand after birth (Gage, et al. 1996a; Gage, et al. 1996b; Nasonkin, et al. 2004; Ward, et al. 2005). Although some aspects of the phenotype observed when Notch2 is lost are similar to that of Prop1 mutants, we do not see increased adhesion and inability of cells to migrate out of Rathke’s pouch in Notch2 cKO mice, as is seen in mice with loss of Prop1 (Ward, et al. 2005). These differences indicate that the levels of Prop1 in Notch2 cKO mice may be sufficient to prevent cell migration defects or that other Notch targets are able to compensate for defects observed when Prop1 is lost.

Genetic causes of CPHD include mutations resulting in hypomorphic SOX2 expression and loss of function mutations in PROP1 and PIT1 (Wu, et al. 1998; Kelberman, et al. 2009; Castinetti, et al. 2012). Our studies show that reductions in Notch signaling can result in alterations in expression of all of these factors during
embryonic and postnatal development. Furthermore, we show that loss of Notch2 can lead to hypoplastic pituitary as early as p6, indicating that the Notch signaling pathway is an important regulator of pituitary size and cell fate. Based on these studies, it may be advisable to consider whether Notch2 or other members of the Notch signaling pathway are altered in patients with CPHD that have no known genetic mutations.

ACKNOWLEDGEMENTS

We are grateful to Dr. Ann Nardulli for use of equipment. Pituitary hormone antibodies were kindly provided by Dr. A.F. Parlow and the National Hormone and Pituitary Program (University of California, Los Angeles). Financial support was received from the National Institute of Health grant T32 GM007283 (To LBN) and R01 DK076647 (LTR).
REFERENCES


## Table 3.1. Primers used for qRT-PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh</td>
<td>GGTGAGGCCGGTGCTGAGTATG</td>
<td>GACCCGTTTTGCCACCATTC</td>
</tr>
<tr>
<td>Dil1</td>
<td>TAACCTCTTGGAGACCTCAGG</td>
<td>TGGCTTGGTGCACGTTC</td>
</tr>
<tr>
<td>Jag1</td>
<td>TGATGAGAAAGCTGTGGTG</td>
<td>TGGTACAGTAGGTTGTTG</td>
</tr>
<tr>
<td>Jag2</td>
<td>TGGAGACCTGAGGAAGAGTAGAAG</td>
<td>ACTGGTCTGGCCTCTCATC</td>
</tr>
<tr>
<td>Notch1</td>
<td>TTCATGATTGCCTCCTGAGTGG</td>
<td>CAGGACTGTGGAGTTGTCGC</td>
</tr>
<tr>
<td>Notch2</td>
<td>TGCCAATCTCCACTCCTGT</td>
<td>TCCACTGACACTGTCCTC</td>
</tr>
<tr>
<td>Notch3</td>
<td>GAGAGCCTTGGCACTCAG</td>
<td>CCACGGAGACAAGCGACAG</td>
</tr>
<tr>
<td>Notch4</td>
<td>GAGGACCTGGGTAAGATGATGAC</td>
<td>TGGCAAGCTGATGAGAGAC</td>
</tr>
<tr>
<td>Hes1</td>
<td>CTCGCTCATTGGGACTC</td>
<td>CTCGCTCATTGGGACTC</td>
</tr>
<tr>
<td>Hey1</td>
<td>CACCGAAGAGCTGACTGCGCACT</td>
<td>CCGCTGACACTGTCCTG</td>
</tr>
<tr>
<td>Hey2</td>
<td>CACCGAAGAGCTGACTGCGCACT</td>
<td>CCGCTGACACTGTCCTG</td>
</tr>
<tr>
<td>Heyl</td>
<td>CACCGAAGAGCTGACTGCGCACT</td>
<td>CCGCTGACACTGTCCTG</td>
</tr>
<tr>
<td>Prop1</td>
<td>CCAGACGGAGGAGGGCTAAG</td>
<td>GGCTATCGGCTGGAGAAGTG</td>
</tr>
<tr>
<td>Sox2</td>
<td>GGAGAAAGAAGAGAGAGAGAGAG</td>
<td>CTGGCAGAGAATAGTGG</td>
</tr>
<tr>
<td>Sox9</td>
<td>AGCCGGACTCCCACTTCCCCC</td>
<td>GAGATTGCCAGAGTCTGCCC</td>
</tr>
<tr>
<td>mKif67</td>
<td>CACCGGATCTCCAGGCCTTCCATTACAG</td>
<td>GGAATGAGCACAGGCCCACATCC</td>
</tr>
<tr>
<td>Ccne1</td>
<td>GGTCACGGACTTTTCTGAGGCCTC</td>
<td>TCGCTGAGAGAAGAGTGAGC</td>
</tr>
<tr>
<td>Cdkn1c</td>
<td>TCCATCACATCATGAGAGGCGCAGAAGGAC</td>
<td>ATCGCTGAGAGGCGCAGAAGGAC</td>
</tr>
<tr>
<td>Ccn1d</td>
<td>CCGCAGTACCATCGACCAAAATC</td>
<td>CTGCCGTCAGACCTCGAAGCTAGG</td>
</tr>
<tr>
<td>Pou1f1</td>
<td>AGGTAGGAGGAGGAGGAGGAGGAGGAG</td>
<td>GGAATGAGTAGGAGGAGGAG</td>
</tr>
<tr>
<td>Gh</td>
<td>AACGAGGAGGAGGAGGAGGAGGAG</td>
<td>GCCCTCAGTGCTCTAGAG</td>
</tr>
<tr>
<td>Tshb</td>
<td>TGTCAGGCTGAGGCACTTCCT</td>
<td>TGGTGGGAGAGGAGTAGAG</td>
</tr>
<tr>
<td>Tpit</td>
<td>GATGTCAGGGAGGAAGAGAG</td>
<td>AGCTTTTCTATCAATTCAG</td>
</tr>
<tr>
<td>Pomc</td>
<td>GTTACGTTGCTTCCCAGTAC</td>
<td>CGGCTTCTATGAGGAGGCTT</td>
</tr>
<tr>
<td>Crhr1</td>
<td>TTTGGTCGGGCACTCGTGAG</td>
<td>AGGTAGGAGGAGGAGGAG</td>
</tr>
<tr>
<td>Pax7</td>
<td>GCCACAGAGAAGAAGGTCAC</td>
<td>TGGTGGGAGAGGAGTAGAG</td>
</tr>
<tr>
<td>Gnrhr</td>
<td>ATGATGGTCGTGATTGAG</td>
<td>ATGATGGTCGTGATTGAG</td>
</tr>
<tr>
<td>Nr5a1</td>
<td>AAATTCTGGAAGACAAACAGAG</td>
<td>GCCATCCTAATGAGAAGGTT</td>
</tr>
<tr>
<td>Lhb</td>
<td>CCCAAGTCACATGATCCTCA</td>
<td>GCCATCCTAATGAGAAGGTT</td>
</tr>
</tbody>
</table>

Table 3.1. Primers used for qRT-PCR analysis.
Figure 3.1. Conditional loss of Notch2 results in decreased Prop1 mRNA at e12.5. Sagittal sections of control (A) and cKO (B) e12.5 pituitaries were stained with hematoxylin and eosin and developing pituitaries appear roughly the same shape and size. Ki67 immunostaining is similar between control (C) and cKO (D) pituitaries. POMC is expressed in few cells in the pituitaries of control (E) and cKO (F) mice. The canonical Notch target Hey1 is expressed in the developing pituitary in control mice (G) and appears reduced in cKO mice (H). Prop1 is expressed in control mice (I) and appears reduced in cKO mice (J). Photos taken at 200x. Scale bar: 50μm. n=4-6.
Figure 3.2. Characterization of mRNA levels of Notch family members in the pituitary during early postnatal development. mRNA levels of Notch ligands (A), receptors (B) and targets (C) were measured at e16.5, p1, p5, p10 and p21 and were compared to levels found in adult pituitaries. *: p< 0.05, significantly increased compared to adult control. #: p< 0.05, significantly decreased compared to adult control. n=4.
Figure 3.3. Localization of Notch family members in the pituitary at p5. *In situ* hybridization for *Notch2* (A and B), *Hey1* (C and D) and *Hes1* (E and F) reveals that Notch signaling components are mainly localized to cells surrounding the pituitary cleft. qRT-PCR comparing control and *Notch2* cKO pituitaries at p1 shows specific and significant reductions in mRNA levels of *Notch2* (G), *Hes1*, *Hey1*, and *Prop1* (H). *:p < 0.05. **:** p < 0.001. Scale bar: 50μm. n=3-4 (in situ hybridization) and n=4 (qRT-PCR).
Figure 3.4. Loss of Notch2 results in misplacement of pituitary progenitors at p1 and decreased progenitor number at p5. SOX2 is expressed at p1 in cells lining the pituitary cleft and in scattered cells in the intermediate lobe (IL) and anterior lobe (AL) of control mice (A, C and E). In contrast, in cKO mice, SOX2 is only present in cells in the dorsal IL and is absent from IL cleft cells (B and D), while AL expression of SOX2 appears to be unaffected in cKO mice (B and F). SOX9 follows a similar expression pattern to that of SOX2 in control mice (G, I and K). SOX9 is restricted to dorsal IL cells (H and J) and appears to be reduced in the AL (H and L) of cKO mice. mRNA levels of Sox2 and Sox9 are reduced in whole pituitaries of cKO mice at p1 as compared to control (M). SOX2 is expressed in cells lining the cleft in the AL and IL in control mice at p5 (N, P, R). In cKO mice, SOX2 containing cells in the IL and the AL are reduced (O, Q, S). In the AL parenchyma, SOX2 cells are seen in clusters in control mice (T) and appear reduced in cKO mice (U). SOX9 is also expressed in cells lining the cleft in the AL and IL in control mice (V, X, Z) and has similar reductions in cKO mice (W, Y, A') as those seen with SOX2. In control mice, SOX9 is seen in clusters in the AL parenchyma (B'). These clusters are not observed in cKO mice (C'). Photos taken at 200x (A, B, G, H, N, O, V, W), and 400x (C-F, I-L, P-U and X-C'). White boxes (A, B, G and H) indicate where higher magnification photos were taken. Arrows (L, S and A') indicate SOX9 and SOX2-negative cleft cells. Scale bars: 25μm (E, F, K, L) and 50μm (A-D, G-J, N-C'). *:p< 0.05. n=6-8 (histology) and n=4-9 (qRT-PCR). AL=anterior lobe. IL=intermediate lobe.
Figure 3.5. Decreased proliferation is observed in mice with conditional loss of Notch2, resulting in a hypoplastic pituitary by p6. Ki67 immunoreactive cells are found in the AL and IL of control (A and C) pituitaries at p1. The number of Ki67-immunopositive cells appears to be reduced in both lobes of cKO (B and D) mice. Phospho-histone H3 (PH3) expression is scattered throughout the pituitary at p1 in control (E) mice and is markedly decreased in cKO (F) pituitaries. qRT-PCR in whole pituitaries at p1 reveals a significant decrease in mRNA levels of mKi67 and Ccne1 and a significant increase in Cdkn1c mRNA in cKO mice as compared to controls (G). Hematoxylin and eosin staining reveals a hypoplastic AL at p6 in cKO mice (J and K) as compared to control mice (H and I). Volume of AL at p6 is decreased in cKO mice. Photos taken at 50x (H-K), 200x (A, B, E and F) and 400x (C and D). White box (A and B) indicates where higher magnification photos were taken. Scale bars: 50μm. *:p< 0.05. ***:p<0.001. n=6-7 (immunohistochemistry), n=4-7 (qRT-PCR) and n=3-4 (AL volume approximation). PL=posterior lobe. AL=anterior lobe. IL=intermediate lobe.
Figure 3.6. Postnatal Notch inhibition results in decreased proliferation and identifies Prop1 as a direct target. Experimental design for in vivo DAPT injection (A). Subcutaneous injection with DAPT results in a decrease in whole pituitary mRNA levels of Hes1, Hey1 and Prop1 as compared to vehicle-injected mouse pituitaries (B). BrdU-positive cells (red) are present throughout the pituitaries of vehicle-treated mice (C, 5.51% ± 0.20%) and are significantly reduced (p=0.0001) in pituitaries of DAPT-treated mice (D, 2.98% ± 0.26%). mRNA levels of mKi67 and Ccnd1 in whole pituitaries are decreased after DAPT injection as compared to vehicle injection (E). Conversely, mRNA levels of Cdkn1c are increased in the whole pituitary of DAPT-injected mice (F). Experimental design for in vitro DAPT treatment and DAPT washout assay (G). After 48h in culture, mRNA levels of mKi67 are significantly decreased compared to vehicle-treated controls (H). mRNA levels of Hes1, Hey1 and Prop1 are reduced after DAPT treatment and recover after 6h DAPT washout (I). Photos taken at 100x. Scale bars: 50μm. *:p< 0.05. **:p<0.01. ***:p<0.001. n=4 (immunohistochemistry), n=5-8 (qRT-PCR in vivo) and n=4-8 (qRT-PCR in vitro).
Figure 3.7. Conditional loss of Notch2 results in decreased expression of Pit1-lineage hormones and increased expression of POMC. GH immunopositive cells are found scattered throughout the AL of control (A) mice and appear to be reduced in pituitaries of cKO (B) at p1. TSHβ is localized to the AL of control (C) and cKO (D) mice. POMC immunoreactive cells are found in the AL and the IL of control (E) pituitaries and appear slightly increased in the AL of cKO mice (F). LHβ expression is confined to the AL and appears similar between control (G) and cKO (H) mice. At p6, GH is present in the AL of control (I) mice and appears reduced in cKO mice (J). Similarly, TSHβ is strongly reduced in cKO mice (L) as compared to control (K). POMC is present in the IL and AL of control mice (M) and appears slightly increased in the AL of cKO mice (N). LHβ appears similar between control (O) and cKO (P) pituitaries. qRT-PCR analysis at p1 reveals a significant decrease in Pou1f1 (Pit1) and Gh mRNA, but no change in Tshb mRNA between control and cKO mice (Q). Additionally, p1 mRNA levels of Tpit and Pomc show a trend towards an increase in cKO mice, whereas Crhr1 is significantly increased and Pax7 is unchanged (R). mRNA levels of Nr5a1, and Lhb are significantly increased in pituitaries, while Gnhrh is trending towards an increase at p1 (S). The percent of Pit1-, POMC- and LHβ-positive cells in the AL was quantified in control and cKO mice and the percent of Pit1-positive cells was found to be decreased in cKO mice, whereas the percent of POMC-immunoreactive cells is increased (T). *: p< 0.05. Photos taken at 200x. Scale bars: 50μm. n=5-7 (histology), n=4-7 (qRT-PCR) and n=3-5 (cell counts). PL=posterior lobe. AL=anterior lobe. IL=intermediate lobe.
Figure 3.8. *Foxg1* Cre mice do not display altered pituitary development or ability to influence hypothalamic Ghrh neuron number. Gh mRNA levels in whole pituitaries of p1 mice with and without a Cre transgene were similar (A). Ghrh mRNA expression is similar in the arcuate nucleus between control (B) and cKO (C) mice at p1. Photos taken at 200x. Scale bars: 50μm. n=3 (in situ hybridization) and n=5-8 (qRT-PCR).
Figure 3.9. Postnatal chemical Notch inhibition results in decreased mRNA levels of *Pou1f1* (*Pit1*), Prl, and Tshb and increased levels of *Pomc*. Experimental design for in vivo subcutaneous DAPT injection (A). mRNA levels of *Pou1f1*, Prl and Tshb are significantly decreased and mRNA levels of *Pomc* are significantly increased in whole pituitaries after DAPT injection as compared to vehicle-treated mice. mRNA levels of Gh and Lhb are unchanged between the two groups (B). Experimental design for in vivo subcutaneous DAPT injection longer paradigm (C). mRNA levels of *Pou1f1* and Prl are decreased in whole pituitaries after DAPT injection as compared to vehicle-treated mice. mRNA levels of all other hormones are unchanged between the two groups (D). *: p< 0.05. n=3-5. **: p< 0.05. n=3-5.
CHAPTER 4: Notch signaling regulates proliferation and cell fate
determination genes in the pituitary

ABSTRACT

During embryonic and postnatal development, pituitary progenitors proliferate and differentiate in response to internal and external cues. Specific factors that are important for regulating these processes remain unclear. Previous work shows that Notch signaling components are elevated during embryonic and postnatal pituitary development and help to regulate proliferation and corticotrope cell number (Chapters 2 and 3). To identify transcriptional targets through which Notch regulates these processes, microarray analysis was performed to compare the pituitaries of control and Notch2 cKO. Gene ontology analysis shows that many of the altered transcripts in Notch2 cKO mice are implicated in cell division events, including mitosis, replication and cell cycle control. Additionally, we identified a subset of genes that are changed in Notch2 cKO mice that are important for corticotrope differentiation and signaling. One such gene, Hsd11b1, which encodes for the 11β-HSD1, is an important factor in regulating local corticosteroid levels, which are known to affect corticotrope number. We confirmed that Hsd11b1 is decreased in Notch2 cKO mice and in mice or pituitary explants treated postnatally with DAPT, a chemical Notch inhibitor. Scanning the Hsd11b1 promoter for candidate regulatory sequences of Notch signaling reveals three Hey1 binding sites, suggesting a mechanism by which the Notch pathway may control expression of this enzyme. Expression analysis shows that Hsd11b1 is expressed at very low levels at e16.5 and
increases by p1. Additionally, we show that 11β-HSD1 is mostly present in POMC-expressing melanotropes and corticotropes. This study is the first to show that Notch signaling can alter Hsd11b1 levels and suggests a mechanism by which Notch may influence corticotrope cell number and pituitary proliferation.

INTRODUCTION

All pituitary endocrine cells are derived from a common pool of progenitors that express the stem cell markers SOX2 and SOX9 (Fauquier, et al. 2008; Andoniadou, et al. 2013; Rizzoti, et al. 2013). During early postnatal development, these progenitor cells remain highly proliferative and integrate intrinsic and extrinsic signals to help them choose between proliferation and endocrine differentiation (Gaston-Massuet, et al. 2011; Gremeaux, et al. 2012). Intrinsic factors that influence postnatal pituitary proliferation include cell cycle control protein Cdk4 (Jirawatnotai, et al. 2004) and Wnt signaling mediator β-catenin (Gaston-Massuet, et al. 2011). Pituitary progenitors can also proliferate in response to external cues. For example, adult mice that have either been adrenalectomized or treated with estrogen have increased pituitary progenitor proliferation (Nolan and Levy. 2006; Rizzoti, et al. 2013). Altered external hormonal signaling can also lead specific changes in differentiated pituitary hormonal cell number. For example, adrenalectomy leads specifically to an increased number of corticotropes (Nolan, et al. 2004). Conversely, treatment with the glucocorticoid receptor agonist, dexamethasone, results in a reduction in corticotrope number (Nolan and Levy. 2006). These changes in corticotrope cell
number are a direct reflection of the pituitary’s ability to regulate cell number in response to signals from both the hypothalamus and the adrenals. In both studies, levels of corticosteroids, which are normally produced by the adrenals are altered, leading to a change in pituitary corticotrope number. Our studies show that Notch signaling is important for both progenitor proliferation and differentiated cell balance, suggesting it may be an important point of integration for intrinsic and extrinsic cues.

Notch signaling is an evolutionarily conserved pathway that functions to maintain cells in a progenitor state by repressing transcription of pro-differentiation factors (Louvi and Artavanis-Tsakonas. 2006). In conjunction with this role, reductions in Notch signaling often result in decreased proliferation (van Es, et al. 2005). In more rare instances, such as in T-cell acute lymphoblastic leukemia, excessive Notch signaling can actively promote proliferation (Pancewicz, et al. 2010). Multiple studies have sought to identify direct Notch targets that may account for Notch’s influence on proliferation. Specifically, Notch signaling has been shown to directly regulate transcription of cell cycle regulatory genes, including \textit{Ccnd1} (Cohen, et al. 2010) \textit{Ccnd3} (Joshi, et al. 2009) and \textit{Cdnk1} (Georgia, et al. 2006). These factors are considered direct targets since the Notch intracellular domain and cofactor, RBPJ, can bind directly to their promoters to influence transcription. Indirect Notch/RBPJ targets, which are controlled by binding of downstream Notch effectors (Hes or Hey proteins) at their promoters, have also been shown to regulate cell cycle modulators (Murata, et al. 2005). In the developing pituitary, loss of the Notch target gene \textit{Hes1}
results in increased expression of the cell cycle inhibitors p21, p27 and p57, suggesting that Notch signaling may control similar factors in the pituitary (Monahan, et al. 2009). Additionally, mice with conditional loss of Notch2 have reduced proliferation and altered levels of cell cycle molecules (Chapter 3). Other factors that are regulated by Notch and are important for pituitary proliferation have yet to be identified.

Notch signaling also regulates cell fate choice in multiple systems, including the pancreas and the nervous system (Ohtsuka, et al. 2001; Ge, et al. 2002; Murtaugh, et al. 2003; Siveke, et al. 2008). In the pituitary, reduced levels of Notch signaling result in an increased number of corticotropes and a decreased number of somatotropes, thyrotropes and lactotropes (Chapter 3). Additionally, when Notch is persistently expressed, differentiation of melanotropes and corticotropes is inhibited (Chapter 2). Identification of novel Notch targets will be important to understand how Notch mediates cell fate choices in the pituitary. The following studies were designed as a preliminary analysis of the transcriptome regulated by Notch, with the goal of determining the mechanism by which Notch influences pituitary proliferation and differentiation.

MATERIALS AND METHODS

Mice

For expression analysis, wildtype male and female mice of mixed genetic background were used. For in vitro and in vivo DAPT treatment studies, CD-1 mice (Charles River, Wilimington, MA), from a breeding colony maintained in our
laboratory, were used. For all studies, p1 is considered to be the day of birth. All animals were housed in a facility with a 12 hour light-dark cycle and were maintained in accordance with the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee.

In order to generate Notch2 cKO mice, Notch2\(^{fl/fl}\) mice were obtained from Jackson Laboratory (Bar Harbor, ME) and bred to Foxg1\(^{+/cre}\) mice, maintained on a 129 background, also purchased from Jackson Laboratory. The resulting Notch2\(^{+/fl}\), Foxg1\(^{+/cre}\) mice were bred to Notch2\(^{+/fl}\), Foxg1\(^{+/+}\) littermates to obtain cKO animals. To genotype the mice, tail biopsies were obtained and DNA was extracted using a hot shot or salt-out method. PCR for the Cre and Notch2 alleles was performed as previously described using published primer sequences (Chapter 3).

**Agilent Array**

Pituitaries of female control and Notch2 cKO p1 mice were collected and stored in RNAlater (Life Technologies, Grand Island, NY) until processing. Two samples from female mice of the same genotype were pooled together and RNA was extracted using the RNAqueous Micro Kit (Life Technologies). RNA was then sent to the Roy G. Carver Biotechnology Center (University of Illinois at Urbana-Champaign), where cDNA was synthesized and spotted on an Agilent 4x44k mouse whole genome array (Agilent Technologies, Santa Clara, CA). Four arrays were run for each genotype. Data analysis was performed by Jenny Drnevich (Bioinformatics Unit, University of Illinois at Urbana, Champaign).
Quantitative Reverse Transcriptase PCR (qRT-PCR)

RNA was isolated from embryonic and postnatal whole pituitaries using an RNAqueous micro kit (Ambion, Austin, TX) as per manufacturers protocol. For p21 and adult pituitaries, 0.5µg of RNA was synthesized into cDNA using the ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA). For e16.5 and p1, p5 and p10 and p12 pituitaries, the total amount of RNA from each pituitary was converted into cDNA. A no Reverse Transcriptase enzyme control was also prepared and used as a negative control. For quantitative RT-PCR 0.1-0.33 µL of cDNA was amplified using gene-specific primers and SYBR green mix (Bio-Rad Laboratories, Hercules, CA) on a Bio-Rad MyIQ real-time PCR machine. Data were analyzed with the change in cycle threshold (ΔCt) value method (Goldberg, et al. 2011), using Gapdh levels to normalize the data. Statistical significance was determined using Student’s t test. The sequences of primers used are as follows: Hsd11b1 forward: TCT TCC ATG ACG ACA TCC AC, Hsd11b1 reverse: GAG TAG GGA GCA ATC ATA GG, Gapdh Forward: GGT GAG GCC GGT GCT GAG TAT G, Gapdh Reverse: GAC CCG TTT GGC TCC ACC CTT C.

DAPT injections

Beginning at p2, CD1 mice were injected daily for 3 days with 100mg/kg DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester, Gamma Secretase Inhibitor IX, Millipore, Billerica, MA) diluted in Dimethyl Sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) or an equivalent volume of DMSO alone. Pituitaries and whole heads were harvested 24h after last injection.
For longer-term studies, mice were injected with 100mg/kg DAPT or an equivalent volume of DMSO for five days beginning on p3. Pituitaries were harvested on p12 or p32.

**Pituitary explant culture and DAPT washout assay**

Pituitaries were dissected from p3 CD1 mice, rinsed with PBS and transferred to a 96 well plate. Culture media was made by adding 1% Penicillin Streptomycin (Fisher, Fairlawn, NJ) and 10% fetal bovine serum (HyClone, Logan, UT) to DMEM/F-12 media (Cellgro, Manassas, VA). 10µM DAPT dissolved in DMSO (Millipore) or an equivalent amount of DMSO (Cambridge Isotope Laboratories, Andover, MA) was added to the culture media from the beginning of culture. After 24h in culture, media was spiked with DAPT or DMSO (an equivalent amount to what was added at the beginning of culture) and pituitaries were cultured for an additional 24h.

**Immunohistochemistry**

Whole heads of p1 mice and pituitaries were fixed using a Zinc fixative (BD, Franklin Lakes, NJ) and embedded in paraffin for sectioning. Slides were deparaffinized and rehydrated. Samples were boiled in 10 mM citrate solution, pH6 for 10 minutes and cooled for 15 minutes. Next, all samples were blocked in a solution containing 5% normal donkey serum (Jackson Immunoresearch, West Grove, PA) diluted in immunohistochemical block, which contains 3% bovine serum albumin (Jackson Immunoresearch) and 0.5% TritonX-100 (Sigma Aldrich, St. Louis, MO) diluted in PBS. Primary antibodies used were raised against the following peptides: 11ß-HSD1 (1:100, Abcam, Cambridge, UK), Pax7
(1:500, Developmental Studies Hybridoma Bank, University of Iowa), Pit1 (1:2000, kind gift of Dr. Simon Rhodes) and POMC (1:1000, Dako, Carpinteria, CA). Slides were then incubated with a biotin-conjugated mouse (Pax7) or rabbit (POMC, Pit1 and 11β-HSD1) secondary antibody. This was followed by incubation in a streptavidin-conjugated cy2 or cy3 fluorophore. Biotin and streptavidin-conjugated antibodies were purchased from Jackson ImmunoResearch and were used at a concentration of 1:500. Slides were counterstained with 4’,6-diamidino-2-phenylindole (DAPI, 1:1000, Life Technologies, Grand Island, NY) and visualized at 50x, 100x, 200x, or 400x magnification using a Leica DM2560 microscope. Photographs were taken using a Retiga 2000R color camera (Q-Imaging, Surrey, Canada) and acquired using Q-Capture Pro software (Q-Imaging). Images were processed using Adobe Photoshop CS4 (San José, CA).

RESULTS

A microarray comparing control and Notch2 cKO p1 pituitaries was performed to identify targets that may contribute to defects observed in proliferation and corticotrope cell number in Notch2 cKO mice. 1310 mRNAs were found to be differentially expressed ($p<0.10$) between control and Notch2 cKO mice. Of these genes, we show that members of the Notch signaling pathway are altered, as expected (Table 4.1). In order to identify pathway or global processes that may be changed, we used DAVID to group the altered genes using gene ontology terms. We find that the majority of top terms changed are related to
proliferation. These include DNA replication, cell cycle and M phase. In addition, we find alterations in transcripts implicated in cell fate commitment and gland development (Table 4.2). Because many of the altered gene products are involved in proliferation, we next examined the array data more closely and identified a subset of cell cycle-associated genes and genes known to influence proliferation that are downregulated when Notch2 is lost (Table 4.3). These genes include Cdca2, Cdca5, Cdca, Ccna1, Ccnf, Pcna and Mki67. These findings are in agreement with our findings showing that overall proliferation and mRNA levels of mKi67 are reduced in Notch2 cKO mice. Future studies validating these changes will be performed in an independent sample set. Additionally, further work will be aimed at determining whether these genes are direct Notch targets in the pituitary.

Additionally, we have identified a subset of differentially expressed genes that may be involved in the regulation of corticotrope differentiation, cell number and/or signaling. These factors include Tbx19, Avpr1b and Crhr1, which are upregulated and Hsd11b1, which is significantly downregulated in Notch2 cKO mice (Table 4.4). Tbx19, Avpr1b and Crhr1 are not generally expressed until after corticotrope differentiation has occurred. Therefore, they may be upregulated as a byproduct of the increase in corticotrope cell number and are not direct factors in the regulation of cell number or differentiation. For this reason, we chose to focus the remainder of this study on how alterations in Hsd11b1, the gene that encodes for 11ß-hydroxysteroid dehydrogenase type 1 (11ß-HSD1), may affect corticotrope cell number and how this gene may be
regulated by Notch. 11β-hydroxysteroid dehydrogenase type 1 is an enzyme that converts inactive cortisone to its active form, cortisol (11-dehydrocorticosterone to corticosterone in mice). This conversion helps to locally increase active cortisol, which could then activate the glucocorticoid receptor. Changes in glucocorticoid signaling have been shown to affect corticotrope cell number during development, (Schmidt, et al. 2009) making this enzyme a possible mechanism by which Notch signaling may influence corticotrope number.

In order to confirm our microarray results showing that Hsd11b1 is downregulated in Notch2 cKO mice, we performed quantitative RT-PCR comparing control and cKO male and female pituitaries at p1. In agreement with the array data, we observe a significant decrease in Hsd11b1 in Notch2 cKO mice (Figure 4.1A). Furthermore, when p3 pituitary explant cultures are treated in vitro with the Notch inhibitor DAPT for 48 hours, a significant reduction in Hsd11b1 is observed (Figure 4.1B). In order to determine if similar effects are observed in vivo, we explored whether Hsd11b1 is altered in several paradigms in which mice were injected with DAPT. In the first paradigm, (shown in chapter 3, figure 3.5A) we show that when mice are injected with 100/mg/kg DAPT on p2, p3 and p4 and then harvested on p5, pituitary mRNA levels of Hsd11b1 are decreased (Figure 1C). In contrast, if mice are injected with DAPT daily from p3 through p7 and harvested on p12 (Figure 4.1D) or p32 (Figure 4.1E), no significant change is observed in Hsd11b1 levels, suggesting levels may be able to recover during the period in which DAPT is not injected. Together, these data,
which examine the effects of both genetic and chemical Notch inhibition, suggest that \textit{Hsd11b1} is a target of Notch signaling.

In order to bioinformatically determine if the Notch cofactor RBPJ or canonical Notch targets have the potential to bind to the promoter of \textit{Hsd11b1}, we scanned the \textit{Hsd11b1} promoter for known RBPJ, Hes1 or Hey1 binding motifs. We identified three Hey1 binding sites within 5kb upstream of the \textit{Hsd11b1} transcriptional start site (Figure 4.2). We did not identify any potential binding regions for either RBPJ or Hes1 using this approach. The discovery of these binding sites implies that \textit{Hsd11b1} may be downstream of Notch signaling and, if so, may be transcriptionally controlled by Hey1. Further work investigating this possibility will be a future area of focus in the Raetzman laboratory.

In addition to investigating whether \textit{Hsd11b1} may be a direct Notch target, we have also begun to examine the expression pattern of \textit{Hsd11b1} during development. This analysis will reveal during which periods the pituitary may be sensitive to alterations in \textit{Hsd11b1} and other glucocorticoid signaling components. We show that the mRNA levels of \textit{Hsd11b1} in the pituitary are very low (near the limits of detection) at e16.5. They rise by p1 to levels similar to those seen in the adult. Interestingly, at p5, a time during which the pituitary is undergoing cell expansion and differentiation, the levels of \textit{Hsd11b1} are significantly increased in comparison to adult. By p9, the levels have decreased to levels similar to those observed in the adult and at p21 the levels are, again, increased (Figure 4.3). These results suggest that \textit{Hsd1b11} begins to become
more highly expressed in the pituitary of mice after birth and is expressed at dynamic levels throughout postnatal expansion.

To determine where in the pituitary $11\beta$-HSD1 is expressed, we performed immunohistochemistry in p1 pituitaries. We find that $11\beta$-HSD1 is expressed in many cells of the IL and in scattered cells of the AL (Figures 4.4A). The expression pattern in the IL appears to closely resemble that of POMC, which is expressed in IL melanotropes. Because these cells express the transcription factor Pax7 (Budry, et al. 2012), we performed a double immunostain to determine whether $11\beta$-HSD1 is expressed in Pax7-expressing cells. We show that $11\beta$-HSD1 is expressed in many, but not all, Pax7-expressing cells and does not appear to be expressed in any Pax7-negative cells in the IL (Figures 4.4B). In order to confirm this observation, we performed colocalization studies with POMC. A similar result was observed in the IL, with $11\beta$-HSD1 present in most, but not all, POMC-expressing melnanotropes (Figure 4.4C). POMC is also expressed in AL corticotropes and we find that the majority of corticotropes express $11\beta$-HSD1 (Figure 4.4D). However, there are many $11\beta$-HSD1-positive cells in the AL that do not colocalize with POMC. To help determine the identity of these cells, we performed double immunohistochemistry for $11\beta$-HSD1 and Pit1, a transcription factor present in somatotropes, thyrotropes and lactotropes. We show that a small subset of Pit1-positive cells also express $11\beta$-HSD1 (Figure 4.4E). These findings show that, at p1, $11\beta$-HSD1 is confined mainly to POMC-producing cells, suggesting it acts directly in cells that are responsive to glucocorticoid signaling.
DISCUSSION

The pituitary contains multiple endocrine cell types that are all derived from a common progenitor population. It is important for function of the gland, and thus of pituitary target organs, that the proper number of each cell type is formed. If too few or too many of any one population is formed, endocrine dysfunction can occur. In Notch2 cKO mice, we observe an increase in the number of POMC-expressing corticotropes. Additionally, microarray analysis revealed a decrease in expression levels of Hsd11b1. Hsd11b1 encodes the 11β-HSD1 enzyme, which converts corticosteroids to their active form, allowing them to bind to and activate the GR. Previous studies have shown that alterations in glucocorticoid signaling can result in changes in corticotrope number. Specifically, when GR is lost in POMC-expressing cells in the pituitary, an increased number of corticotropes is observed at p6 (Schmidt, et al. 2009). Additionally, after adrenalectomy in adult rats, an increase in proliferation and corticotrope number is observed (Nolan, et al. 2004). Conversely, when adrenalectomized rats are treated with dexamethasone, a GR agonist, an increase in pituitary apoptosis and a decrease in pituitary proliferation are observed (Nolan and Levy. 2006). These data show that the pituitary is able to regulate cell number in response to alterations in GR signaling and that the corticotrope POMC cell lineage is especially sensitive to these alterations. These data may help to explain the phenotype of increased corticotrope number in Notch2 cKO mice, as these mice likely have decreased signaling through the GR due to decreased levels of Hsd11b1.
11β-HSD1 is a key metabolic enzyme whose dysregulation has been implicated in obesity and type 2 diabetes. Mouse models with overexpression of 11β-HSD1 develop many features of metabolic syndrome, including obesity, glucose insensitivity, insulin resistance and hyperlipidemia. Humans with metabolic syndrome also exhibit increased levels of 11β-HSD1. Because of these observations, 11β-HSD1 is a promising target for the treatment of metabolic syndrome and multiple studies have explored the effectiveness of 11β-HSD1 inhibitors in the clinic [reviewed in (Pereira, et al. 2012)]. Although much research has been focused on how this enzyme is dysregulated in metabolic syndrome and how it may be a useful target to combat such problems, little is known about how 11β-HSD1 expression regulated. We present data showing that mice with reduced Notch signaling have significantly reduced levels of Hsd11b1, suggesting this pathway may be involved in its regulated expression. There is evidence to suggest that, in other systems, levels of Notch signaling can influence metabolic processes. Previous studies have shown that both hypo- and hyper- activated Notch signaling induce a glycolytic phenotype in breast cancer cells (Landor, et al. 2011) and persistent expression of Notch1 promotes insulin resistance and induces expression of glucose-6-phosphatase (Valenti, et al. 2013). These studies show that Notch signaling has the ability to regulate multiple metabolic processes and contribute to the notion that Notch may regulate metabolic enzymes in the pituitary.

We have identified three Hey1 binding sites within 5kb upstream of the Hsd11b1 transcriptional start site. These identified sites indicate that Notch target
gene products may have the ability to regulate directly the expression of
Hsd11b1. Hey1 and other canonical Notch downstream effectors have been
categorized as transcriptional repressors. Therefore, if Hey1 regulates
Hsd11b1 directly, it would be expected that a reduction in Hey1 would result in
an increase in Hsd11b1 mRNA levels. Surprisingly, we observe a decrease in
Hsd11b1 levels when Notch2 is lost and Hey1 is downregulated. This
relationship suggests that if Hey1 were to directly regulate Hsd11b1, it may be
acting as a transcriptional activator. Although uncommon, previous studies have
identified activation roles for Hey1. A study that utilized microarray and chromatin
immunoprecipitation identified a subset of targets that are activated by Hey
proteins (Heisig, et al. 2012). Additionally, Hey1 has been implicated in the
activation of p53, although the mechanism remains unclear (Huang, et al. 2004;
Ban, et al. 2008). Transcriptional activation roles for Hes factors, which are also
Notch targets and members of the basic helix-loop-helix family, have also been
described. Specifically, in human fibroblast cells, Hes1 activates transcription of
the human acid-glucosidase (GAA) gene and Hes1 and Hes5 have the ability to
activate transcription of STAT-3 (Kamakura, et al. 2004). These studies provide
precedent for the ability of Notch canonical targets to activate other genes.

Our data shows that 11β-HSD1 is expressed mainly in corticotropes and
melanotropes. Previous findings show that Notch signaling components,
including Hey1, are expressed in cells surrounding the pituitary cleft and in
scattered AL cells, in a pattern mirroring that of the progenitor markers SOX2 and
SOX9 (Chapter 3). Because Notch signaling is not likely active in melanotropes
and corticotropes, it is unlikely that the downstream Notch effector, Hey1 is able
to directly regulate Hsd11b1 expression. Future studies will be necessary to
identify a target which is both under direct Notch control and that can directly
control corticotrope number.

11β-HSD1 is an important metabolic regulator that has been implicated in
the development and progression of metabolic syndrome. Therefore,
understanding how this enzyme is controlled is of great importance. We show
that reductions in Notch signaling results in decreased levels of Hsd11b1,
suggesting that the Notch signaling pathway may be involved in its regulation.
Future studies in the Raetzman laboratory will be aimed at determining whether
Hsd11b1 is a direct target of Notch signaling and how alterations in this enzyme
and other glucocorticoid signaling factors regulate corticotrope cell number
during early postnatal pituitary expansion.

**ACKNOWLEDGEMENTS**

The data presented in this chapter were obtained with technical assistance from
Whitney Edwards.
REFERENCES


Table 4.1. Notch ligands, targets and cofactors changed in Notch2 cKO mice, as identified by microarray analysis. Four independent samples from control and cKO mice were used to determine significance.

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Gene</th>
<th>Gene Name</th>
<th>Fold Change</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctn1</td>
<td>contactin 1</td>
<td>1.739</td>
<td>0.0220</td>
<td></td>
</tr>
<tr>
<td>Dll3</td>
<td>delta-like 3</td>
<td>1.427</td>
<td>0.0231</td>
<td></td>
</tr>
<tr>
<td>Jag2</td>
<td>jagged 2</td>
<td>1.423</td>
<td>0.0306</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Targets</th>
<th>Gene</th>
<th>Gene Name</th>
<th>Fold Change</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dtx4</td>
<td>deltex 4</td>
<td>-1.435</td>
<td>0.0354</td>
<td></td>
</tr>
<tr>
<td>Hey1</td>
<td>hairy/enhancer-of-split related with YRPW motif 1</td>
<td>-2.045</td>
<td>0.0499</td>
<td></td>
</tr>
<tr>
<td>Dtx1</td>
<td>deltex 1</td>
<td>1.327</td>
<td>0.0665</td>
<td></td>
</tr>
<tr>
<td>Hes1</td>
<td>hairy and enhancer of split 1</td>
<td>-1.293</td>
<td>0.0810</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cofactors</th>
<th>Gene</th>
<th>Gene Name</th>
<th>Fold Change</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rbpjl</td>
<td>recombination signal binding protein for immunoglobulin kappa J region-like</td>
<td>-1.234</td>
<td>0.0744</td>
<td></td>
</tr>
</tbody>
</table>
Gene Ontology (GO) Terms Identified as Changed in *Notch2 cKO Mice*

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Number of Changed Genes</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Cycle</td>
<td>81</td>
<td>1.4x10^{-13}</td>
</tr>
<tr>
<td>DNA replication</td>
<td>30</td>
<td>2.7x10^{-9}</td>
</tr>
<tr>
<td>M Phase</td>
<td>40</td>
<td>8.0x10^{-8}</td>
</tr>
<tr>
<td>Cell Fate Commitment</td>
<td>18</td>
<td>0.0027</td>
</tr>
<tr>
<td>Gland Development</td>
<td>20</td>
<td>0.011</td>
</tr>
</tbody>
</table>

Table 4.2. Pathways, grouped by gene ontology term, found altered in *Notch2 cKO* mice. Analyzed using DAVID (Database for Annotatin, Visualization and Integrated Discovery) bioanalysis software.
Table 4.3. Proliferation factors changed in Notch2 cKO mice, as identified by microarray analysis. Four independent samples from control and cKO mice were used to determine significance.
**Corticotrepe/Melanotrope Differentiation or Signaling**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Name</th>
<th>Fold Change</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hsd11b1</em></td>
<td>hydroxysteroid 11-beta dehydrogenase 1</td>
<td>-2.851</td>
<td>2.88x10^-16</td>
</tr>
<tr>
<td><em>Tbx19</em></td>
<td>T-Box 19</td>
<td>1.540</td>
<td>0.0231</td>
</tr>
<tr>
<td><em>Avpr1b</em></td>
<td>arginine vasopressin receptor 1B</td>
<td>1.769</td>
<td>0.0354</td>
</tr>
<tr>
<td><em>Crhr1</em></td>
<td>corticotropin releasing hormone receptor 1</td>
<td>1.389</td>
<td>0.0796</td>
</tr>
</tbody>
</table>

Table 4.4. Corticotrepe/Melanotrope factors changed in *Notch2* cKO mice, as identified by microarray analysis. Four independent samples from control and cKO mice were used to determine significance.
Figure 4.1. Reductions in Notch signaling result in a decrease in Hsd11b1 mRNA levels. Mice with conditional loss of Notch2 (Notch2 cKO) have significantly decreased pituitary mRNA levels of Hsd11b1 at p1 as compared to control (A). p3 pituitary explants treated with in vitro with 10µM DAPT have significantly reduced mRNA levels of Hsd11b1 as compared to DMSO controls (B). Mice injected subcutaneously with 100mg/kg DAPT from p2 through p4 and dissected 24 hours later show a significant reduction in Hsd11b1 pituitary mRNA levels as compared to control (C). Mice injected with 100mg/kg DAPT from p3-p7 and harvested on p12 (D) or p32 (E) do not show differences in pituitary mRNA levels of Hsd11b1. n=3-8. ***:p<0.001.
Figure 4.2. Schematic showing positions of Hey1 consensus binding sites within 5kb upstream of the \textit{Hsd11b1} transcriptional start site.
Figure 4.3. *Hsd11b1* mRNA begins to be more highly expressed in the mouse pituitary after birth and is dynamic throughout postnatal development. All levels are compared to adult. n=3-4. *: p<0.05.
Figure 4.4. 11β-HSD1 (red) is expressed in melanotropes, corticotropes and few Pit1 I positive cells. Coronal section showing 11β-HSD1 expression in the IL and AL of wildtype mice (A). Most IL melanotropes contain Pax7 and some, but not all also express 11β-HSD1 (B). 11β-HSD1 is expressed in many POMC-containing melanotropes (C). Many, but not all, corticotropes express 11β-HSD1 (D). Few Pit1 cells contain 11β-HSD1 (E). Arrows indicate colocalized cells. Photos taken at 200x (A) and 400x (B-E). Scale bar: 50μm. n=2-3.
CHAPTER 5: Conclusions/Discussion

The pituitary sits at the center of the endocrine system, acting as a relay between the hypothalamus and various target organs. Improper development or function of the pituitary can lead to many disorders, including combined pituitary hormone deficiency (CPHD), Cushing’s syndrome, isolated ACTH deficiency, acromegaly and dwarfism (Daly, et al. 2006; Drouin, et al. 2007; Castinetti, et al. 2012). An understanding of how cell number and cell fate are regulated in the pituitary is essential to our understanding of these disorders, as many of them are characterized by inappropriate cell number or absence of one or more endocrine cell types. The studies described in Chapters 2 through 4 have greatly contributed to the understanding of how Notch signaling regulates pituitary cell fate and cell number and how alterations in Notch signaling can lead to pituitary dysfunction. These findings are summarized in figure 5.1.

Notch signaling is an evolutionarily conserved pathway that functions to maintain cells in a progenitor state (Kopan and Ilagan. 2009). In many systems it has also been shown to regulate the choice between differentiated cell lineages, most notably in the nervous system where it influences the choice between neurons and glia (Ge, et al. 2002; Patten, et al. 2003). Studies have shown that Notch signaling components are expressed in the developing pituitary as early as e10.5 (Raetzman, et al. 2004; Zhu, et al. 2006). Mice with loss of the Notch effector Hes1 have reduced pituitary proliferation and absence of the melanotrope lineage (Raetzman, et al. 2007; Monahan, et al. 2009). When Hes1 is lost in conjunction with Prop1, early corticotrope differentiation is observed
(Himes and Raetzman. 2009). Conversely, Notch2 prevents differentiation when overexpressed in gonadotropes (Raetzman, et al. 2006). These studies provide insight into the roles of Notch during embryonic pituitary development. The present studies seek to build upon and expand these observations by examining how Notch signaling acts to regulate proliferation and cell specification in various temporal and cell type-specific scenarios. Specifically, we show that persistent expression of activated Notch inhibits the differentiation of melanotropes and corticotropes and results in dysfunction of the hypothalamic-pituitary-adrenal axis (Goldberg, et al. 2011) Chapter 2). Additionally, we show that Notch signaling is necessary for postnatal pituitary proliferation, progenitor maintenance and cell fate choice (Chapter 3). In order to mechanistically understand how Notch influences these processes, we have identified targets of Notch signaling in the pituitary and have begun to interpret how these factors function in the pituitary (Chapter 4).

**Persistent Notch signaling prevents the differentiation of corticotropes and melanotropes** (Goldberg, et al. 2011)

Isolated adrenocorticotrophic hormone (ACTH) deficiency is a rare condition in which corticotropes either fail to form or fail to produce ACTH. It can be caused by mutations in the *TPIT (TBX19)* gene and the congenital form of the disease is often fatal before diagnosis is made (Pulichino, et al. 2003; Vallette-Kasic, et al. 2004). We show that constitutive expression of activated Notch in melanotropes and corticotropes inhibits their differentiation and causes a phenotype similar to that seen in patients with isolated ACTH deficiency.
Because of this, it may be important to screen patients with ACTH deficiency to determine whether mutations in regulatory regions of Notch may be involved in this disease. Furthermore, this mouse model may be a good system in which to study isolated ACTH deficiency, as it closely mimics the human condition.

A major finding of this study is the observation that melanotropes and corticotropes fail to regenerate when ablated by persistent Notch expression. We show that by p7, the melanotrope-specific transcription factor Pax7 (Budry, et al. 2012) is no longer expressed and that by p30, the IL is reduced to a very thin layer of cells containing only progenitor cell markers. Additionally, we do not observe POMC expression in the AL, suggesting a similar elimination of the corticotrope population. Interestingly, recent studies have shown that the melanotrope population appears to mainly regenerate from progenitor cells, whereas corticotropes regenerate using self-duplication (Langlais, et al. 2013). In our model, progenitor cells are not affected, yet we still observe failure of melanotropes to form and/or expand. It is possible that this is due to rapid activation of Notch in newly born melanotropes, which may prevent them from differentiating. Future studies aimed at understanding how hormone cells regenerate from progenitor cells and/or self-regenerate will be important to our understanding of the differences observed in these models.

**Notch2 is necessary for postnatal proliferation, progenitor maintenance and cell differentiation**

This study is the first to identify a specific signaling pathway that is important during postnatal pituitary expansion. Although studies have shown how
pituitary proliferation and progenitor number change during the first three postnatal weeks in mice (Carbajo-Pérez and Watanabe. 1990; Gaston-Massuet, et al. 2011; Gremeaux, et al. 2012), no study has examined which factors are in regulating these processes. Mice with loss of Notch2 at the onset of embryonic pituitary development do not display defects until p1. These mice have a progressive loss of progenitor cells in the postnatal period, accompanied with a reduction in overall pituitary proliferation. Previous studies have shown that the number of progenitor cells is elevated during the postnatal period (Carbajo-Pérez and Watanabe. 1990; Gaston-Massuet, et al. 2011; Gremeaux, et al. 2012). This increased number of progenitors may be necessary to support the large expansion that occurs in the pituitary shortly after birth in mice. During this time, it is hypothesized that the pituitary begins to respond to external signaling, which is thought to influence cell proliferation and specification. We show that Notch signaling has a role in maintaining progenitor number during the postnatal period, which is not surprising given the large body of work showing that Notch signaling is an important progenitor maintenance factor (Ehm, et al. 2010; Aujla, et al. 2013). It is proposed that these progenitor cells give rise to intermediate progenitors, which are responsible for the majority of the proliferation in the pituitary. These intermediate progenitors are then thought to differentiate into endocrine cells. We observe a reduction in proliferation and which indicates there may be a deficit in number or proliferation of this putative intermediate progenitor population. Defects in different progenitor populations may also contribute to the differences in endocrine cell number that is observed. Future studies aimed at
identifying a marker of these intermediate progenitor populations and determining how they contribute to postnatal pituitary expansion will be essential to our understanding of postnatal pituitary development and the pathways that are important during this developmental window.

An important finding of this study is that Notch2 is dispensable for embryonic proliferation. Previous studies have shown that global loss of the Notch effector Hes1 results in a decrease in pituitary proliferation (Monahan, et al. 2009). However, it is unclear from this study whether the defect in proliferation is intrinsic to the pituitary or caused by loss of Hes1 in the surrounding tissues, namely the hypothalamus which releases signals that are important for pituitary development and proliferation (Treier, et al. 1998; Ericson, et al. 1998). Our findings suggest that the latter may be true. In agreement with this conclusion, mice with pituitary-specific loss of the Notch cofactor Rbpj also show no alterations in proliferation at e14.5(Zhu, et al. 2006). Further studies examining mice with pituitary-specific and hypothalamic-specific loss of Hes1 will necessary to determine how this factor regulates pituitary proliferation.

We also show that Notch2 helps to regulate the balance of differentiated cells in the developing pituitary. Specifically, inhibition of Notch leads to an increase in corticotropes and a decrease in cells of the Pit1 lineage. When Notch is chemically inhibited in postnatal mice, similar results are found in Pomc and Pit1 mRNA levels, suggesting this role is maintained throughout postnatal development. These findings are in agreement with studies showing that mice with pituitary specific loss of Rbpj have decreased Pit1 expression (Zhu, et al.
persistent Notch signaling inhibits the differentiation of corticotropes. This study indicates that Notch singling is necessary to inhibit corticotrope differentiation and promote Pit1 lineage expansion, acting as a choice point between these two lineages. These effects may be direct, with Notch signaling acting in differentiating cells to influence their specification. Additionally, Notch may also act in progenitor cells to bias them towards distinct intermediate progenitor populations.

**Identification of Potential Notch Targets**

In chapter 3, we show that Notch signaling is necessary for postnatal pituitary proliferation and progenitor maintenance. In chapters 2 and 3, we also highlight a role for Notch signaling in inhibiting the corticotrope lineage and promoting the Pit1 lineage. Previous studies have shown that Notch signaling may transcriptionally regulate Prop1, which is necessary for transcription of Pit1, and thus for GH, TSH and Prl hormone synthesis. We developed a DAPT washout assay and used it to validate Prop1 as a bona fide Notch target. This finding is important, as human mutations in the first intron of PROP1, where an RBPJ consensus sequence is found, can lead to CPHD (Godi, et al. 2012). The confirmation that Notch signaling likely regulates Prop1 expression provides clues as to how a pathway, like Notch, that is necessary for many aspects of embryonic development, can have a tissue specific effect.

In order to extend the findings in Chapters 2 and 3 and to identify novel Notch targets in the pituitary, we performed a microarray comparing control and
Notch2 cKO pituitaries. Many of the genes identified by this array are involved in various aspects of cell proliferation. This includes factors that are involved in mitosis, cell cycle progression and replication. We also identified changes in genes involved in pituitary cell differentiation. Of these we identified changes in factors that may regulate corticotrope number. These genes are of specific interest since we show Notch is important to inhibit corticotrope differentiation.

Specifically, our array revealed a significant reduction in Hsd11b1 when Notch2 is lost in the pituitary. The Hsd11b1 gene encodes for the protein 11β-HSD1, which is necessary for the conversion of inactive corticosteroids to their active forms. In late prenatal and early postnatal development, the pituitary is thought to begin to respond to external cues from the hypothalamus and its target organs. Reductions in Hsd11b1 would decrease effective glucocorticoid signaling in the pituitary, as there would be a reduction in active corticosteroids, which bind to and activate the glucocorticoid receptor (GR). Loss of the GR in the pituitary results in an increase in corticotrope number, whereas an increased GR leads to decreased ACTH production (Reichardt et al, 2000, Schmidt et al, 2009). We hypothesize that reductions in Hsd11b1 levels in Notch2 cKO mice lead to decreased glucocorticoid signaling and increased corticotrope number.

Although Notch has been most studied for its roles in progenitor maintenance and cell fate choice, there is precedent to believe that Notch signaling is crucial for the regulation of metabolic processes, including insulin sensitivity and glycolysis (Landor et al, 2011, Valenti et al, 2013). Understanding the extent to which Notch controls Hsd11b1 and how this factor is important for
pituitary development and function are important new areas of research in our laboratory. Furthermore, we are investigating the possibility that of whether Notch influences expression of *Hsd11b1* in other important metabolic organs, such as the liver. These studies will be another area of focus in the Raetzman laboratory and will contribute to a more global understanding of *Hsd11b1* regulation and function.

**Overall Conclusions**

The studies presented in Chapters 2-4 have greatly expanded our understanding of the role of Notch signaling throughout pituitary development. Specifically, this work is the first to define a role for this pathway in postnatal pituitary expansion. In doing so, we have identified the first signaling pathway to be active during this period, which is an emerging area of research in the pituitary development field. Each of the mouse models presented throughout this work represent valuable tools to further explore remaining questions. These questions include, but are not limited to, how isolated ACTH deficiency affects development of other cell types in the pituitary, how progressive loss of pituitary progenitors can lead to hypopituitarism and how glucocorticoid signaling alterations can affect corticotrope cell number. These areas of research are potential avenues that can be explored in the future.
REFERENCES


Drouin, J., Bilodeau, S., Vallette, S., 2007. Of Old and New Diseases: Genetics of Pituitary ACTH Excess (Cushing) and Deficiency. Clinical genetics 72, 175-82.


Figure 5.1 Summary of key findings. Our studies show that Notch2 is necessary for postnatal progenitor maintenance and proliferation. Additionally, gain and loss of function models reveal that Notch signaling promotes the Prop1/Pit1 lineage and inhibits the corticotrope/melanotrope lineage, thus regulating differentiated cell number in the developing pituitary.