INCREASED TSH-PRODUCING CELLS IN THE PITUITARY GLAND OF *Pax6* HAPLO-INSUFFICIENT MICE

By

KENJI JOHNSON

(Under the Direction of James D. Lauderdale)

ABSTRACT

Pax6 is a highly conserved transcription factor that is critical for central nervous system development. It is expressed in the eye, forebrain and hindbrain, and spinal cord. It is also expressed in the endocrine cells of the pancreas and GI tract, the islets of Langerhans and enteroendocrine cells, respectively. Many of these functions have been reported by studying the effects of a complete absence of *Pax6*. However, the complete absence of *Pax6*, in both humans and mice results in the absence of eyes, deformed nasal cavities, and neonate lethality. *Pax6* is semidominant such that a mutation in one copy of the gene results in a congenital condition known as Aniridia in humans. Aniridia is defined by the absence of the iris and the ocular phenotypes of this haplo-insufficiency in *Pax6* have been well described. However, less is known about how a heterozygous mutation in *Pax6* affects other places where *Pax6* is expressed. Recently, Aniridic patients have reported an increased propensity for obesity, infertility, polycystic ovarian disease, and severe eczema. Additionally, several lines of evidence suggest an impact on the brain as well as the gut. In this dissertation, we have attempted to illuminate the impact of *Pax6* haplo-insufficiency on the endocrine system, including the previously unexplored impact on the pituitary gland and enteroendocrine cells, as well as in the brain. We

found that a haplo-insufficiency in *Pax6* results in a decrease in GH-producing cells and increase in TSH-producing cells in neonate mice, with the increased TSH-producing cells continuing in adult pituitary glands. This increase in TSH-producing cells results in an increase in circulating levels of T_4, although no significant change in circulating T_3 levels were found. We also found that a haplo-insufficiency results in structural changes in the adult cerebellum, hippocampus, olfactory bulb, and slight changes in the anterior commissure. Together, these data show that a haplo-insufficiency in *Pax6* impacts development and function of a number of organs where *Pax6* is expressed and reveals a role for *Pax6* in the endocrine system that has previously been unexplored and provides evidence for further studies for the impact of *Pax6* haplo-insufficiency.

INDEX WORDS:

Pax6, paired homeobox six; endocrine system; TRH, thyroid releasing hormone; CRH, corticotropin releasing hormone; SST, somatostatin; GHRH, growth hormone releasing hormone; DA, dopamine; GnRH, gonadotropin releasing hormone; POMC, proopiomelanocortin; ACTH, adrenocorticotropin; MSH, melanocortin stimulating hormone, OT, oxytocin; AVP, arganine vasopressin; GH, growth hormone; PRL, prolactin; TSH, thyroid stimulating hormone; LH, leutenizing hormone; FSH, follicle stimulating hormone; T_4, thyroxin; T_3, triiodothyronine; GLP, glucagon-like peptide; GIP, gastric inhibitory peptide; PC, prohormone convertase; VBM, voxel-based morphometry; MRI, magnetic resonance imaging.

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B.A., The University of North Carolina – Chapel Hill, 2005

A Dissertation Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2016

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May 2016

DEDICATION

I dedicate this dissertation to my Lord and Savior, Jesus Christ, as well as my family. I especially thank my mother, for her continued persistence to continue my education after receiving my BA, my father for his support, guidance, and understanding, my brothers, Derrick and Jon, for their unending encouragement and confidence in me, and to my niece, whose very presence gives me the push to keep going when I feel like giving up. I also dedicate this to my grandparents, who, despite their lack of and/or little education, worked hard in being able to provide a better life for their children, which has allowed me to see a PhD as a feasible goal.

ACKNOWLEDGEMENTS

I would like to first acknowledge and thank my primary advisor, Dr. James D. Lauderdale, for believing in me as a student and scientist even when I did not believe in myself. I also acknowledge and thank him for guiding and allowing me to have the scientific creativity to plan and execute this project with a great deal of independence. I would also like to thank my committee members, Dr. Cordula Schulz, Dr. Douglas Menke, Dr. Brian Condie, and Dr. Tamas Nagy for their continued guidance, suggestions, and discussions on these projects as well as future endeavors. I would also like to thank Dr. Scott Dougan for his encouragement and support, both as a professor and graduate coordinator, Dr. Marcus Fechheimer for his encouragement, particularly during my first two years as a graduate student, and Dr. Jonathon Eggenschweiler for continuously sharing great ideas and feedback during lab meetings.

I would also like to thank a host of research scientists and post-docs in neighboring labs, including: Dr. Julie Gordon for help and guidance in experiments, manuscript preparation, and scientific discussion, serving as a surrogate PI in times when my own was busy, Dr. Kristina Buac and Dr. Jena Chojnowski, who also provided invaluable help and support to me as a student, and Dr. Carlos Infante and Dr. Sungdae Park. I would also like to thank Dr. Cynthia Ward at the UGA vet school for helpful discussion regarding the endocrine tests.

Finally, I would like to thank both past and current members of the Lauderdale and Menke lab, who became like a family as we spent so much time together, including Vani Hariharan, Dr. Alexandra Mihala, Dr. Lindsey Beebe, Dr. Vijay Kalaskar, Anastassia Bobilev, Ariel VanLeuven, and Madison Grant.

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CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

The Endocrine System

The vertebrate endocrine system provides homeostasis of a number of physiological processes via communication between several glands and organs throughout the body. This communication system is similar to the nervous system, but instead of neurotransmitters, makes use of hormones that are released into the bloodstream to communicate. This vast and effective communication system includes parts of the brain, several glandular organs such as the pituitary, thyroid gland, and adrenal glands, and non-glandular organs such as the liver, gonads, pancreas, and intestines. The hormones that are produced by these organs are released directly into the bloodstream, unlike many digestive hormones, for example, that are released into ducts that then empty into the bloodstream, making up the exocrine system.

The endocrine system includes the hypothalamus, whose hormones act on the pituitary gland. Upon stimulation from the hypothalamus, the pituitary then releases hormones that act on specific organs within the body. Many of these organs also release hormones themselves to exert the final effect on the body as well as represent the final point in regulation of homeostasis. Other organs that are not directly acted upon by the hypothalamus include the pancreas and the small and large intestines. Each regulatory organ that is part of the endocrine system entails a great deal of complexity and will require its own explanation. These explanations will include the development and function of the respective regulatory endocrine organ and/or region. Within these sections, the role of *Pax6* in its development and function will also be discussed.

Hypothalamus

The hypothalamus is a defined region of the brain located at the basal part of the dienephalon, directly below the thalamus and directly above the median eminence of the pituitary gland. It forms the wall of the third ventricle and includes the optic chiasm, mammilary bodies, the infundibulum of the pituitary gland, and the tuber cinereum (the part of the diencephalic floor that ends towards the infundibulum) (1). In addition to its role in regulating homeostasis, such as hunger and thirst, the hypothalamus is considered the master regulator of the endocrine system. The neuro-hormones that it releases, known as trophic hormones, start a cascade of hormone release, first to the pituitary gland, and then to a variety of organs throughout the body to regulate a number of bodily functions, including growth, metabolism, reproduction, and development, among other things (Figure 1.1). As part of this system, higher brain centers communicate with the hypothalamus by synaptic contacts, which it then interprets and responds to by releasing the appropriate hormones to the pituitary gland. Hypothalamic hormones are released directly into blood vessels that lead to the tuber cinereum region, whose numerous blood vessels drain into the pituitary stalk. The contents of vessels within the pituitary stalk then empty into the median eminence, the final end of the portal system that connects the central nervous system to the pituitary gland.

The hypothalamic hormones released are primarily named for the pituitary hormone that it regulates and include: corticotropin releasing hormone (CRH) which stimulates release of adrenocorticotropin hormone (ACTH), growth hormone releasing hormone (GHRH) and somatostatin (SST), which stimulates and inhibits release of growth hormone (GH), respectively, thyroid releasing hormone (TRH), which stimulates release of thyroid stimulating hormone (TSH), and gonadotropin releasing hormone (GnRH), which stimulates release of leutenizing

hormone (LH) and follicle stimulating hormone (FSH). While there is no known factor that stimulates release of prolactin (PRL), hypothalamic dopamine (DA) inhibits its release from the pituitary gland. DA exerts the same effect on release of melanocortin stimulating hormone (MSH) from the intermediate pituitary gland. Finally, arganine vasopressin (AVP) and oxytocin (OT) cell bodies are found in the hypothalamus, and the axons extend down into the posterior pituitary lobe.

The hypothalamus is comprised of various clusters of neurons that symmetrically surround the third ventricle. These regions include the suprachasmatic nucleus, arcuate nucleus, anterior hypothalamic nucleus, paraventricular nucleus, periventricular nucleus, dorsomedial nucleus, ventromedial nucleus, supraoptic nucleus, and, although not derived from the same region in development, it also includes the preoptic area (Figure 1.2). The paraventricular nucleus and supraoptic nucleus contain the OT and AVP cell bodies, whose axons extend into the posterior pituitary gland, as well neurons that release TRH and CRH. Some TRH-releasing neurons can also be found in the preoptic area. GHRH neurons are found mainly in the arcuate and ventromedial nuclei while SST neurons are located in the periventricular region. Finally, GnRH neurons are found mainly in the preoptic area in rodents, but in humans they are found mainly in the arcuate nucleus (Figure 1.2).

The hypothalamus has traditionally been thought to form as a part of the diencephalon. As part of the prosencephalon, or forebrain, the primary vesicle of the brain divides in two to form the telencephalon and diencephalon, followed by the generation of the primordial hypothalamus from the ventro-lateral wall of the diencephalon (2). Recently, however, a new model has been proposed in which the hypothalamus develops from the telencephalon (2). Still, in both models, there are several extrinsic and intrinsic factors that are important for

development of the hypothalamus. Of these, the extrinsic factor sonic hedgehog (*Shh)* has been suggested to be essential for early hypothalamic induction (3). It is initially expressed in the prechordal mesoderm and once the hypothalamus has formed, *Shh* expression is restricted to the hypothalamic neuro-epithelium. Here, it controls growth and patterning of the basal hypothalamus and specification of the lateral hypothalamic region by functioning as a signaling center for precursor cells surrounding the basal and lateral regions. Indeed, mouse mutants lacking *Shh* have a hypoplastic basal hypothalamus and lack of specification of the lateral region (4). Additionally, birthdating studies revealed that *Shh* expressing precursor cells give rise to cells in the preoptic area and lateral hypothalamus, only a few cells within the mammillary bodies and ventral midline, and none in the anterior hypothalamic region (5).

Of the intrinsic factors involved in development of the hypothalamus, *Otp, Sim1*, and *Arnt2* have been shown to be critical for its development. The homeodomain-containing orthopedia gene (*Otp*) contributes to the patterning of the preoptic area of the hypothalamus and differentiation of CRH, OT, and AVP neurons in the paraventricular nuclei, as well as other neurons within the supraoptic, arcuate, and ventromedial nuclei, including SST and DA neuorns (6,7). *Otp* null mutant mice have reduced cell proliferation and migration in the hypothalamic region and fail to terminally differentiate neurons in the paraventricular, supraoptic, and arcuate nuclei regions.

Basic helix-loop-helix family members *Sim1* and *Arnt2* are also critical for hypothalamic development. Along with *Otp,* these two transcription factors activate *Sim2* expression in the paraventricular nucleus to allow terminal differentiation of TRH and SST neurons (8). Furthermore, their expression in the paraventricular and supraoptic nuclei works to maintain expression of *Brn2*, which is required for development of oxytocin, arganine vasopressin, and

CRH producing neurons (9). Mice null for both *Sim1* and *Arnt2* show decreased efficiency in neuronal migration and differentiation within the two regions they are expressed. Finally, the orphan nuclear receptor steroidogenic factor 1 (SF1) is also important for development of the hypothalamus. Although its expression is also found in the gonads, anterior pituitary gland, and adrenal cortex, in the hypothalamus it is found in the ventromedial nuclei where it interacts with leptin in regulating body weight homeostasis (10). Furthermore, mutant mice with an absence of one or both copies of SF1 have an abnormal stress response (11,12).

As mentioned earlier, the hormones of the hypothalamus are primarily named for the pituitary hormone they interact with. CRH-producing neurons co-express the posterior pituitary AVP, which acts synergistically with CRH to release ACTH. In addition to the circulating levels of glucocorticoids, stress and the release of catecholamines are strong stimulators of CRH release. If there is not enough or too much circulating glucocorticoids, it can inhibit CRH release as well. GHRH works along with SST to regulate GH release from the anterior pituitary in a pulsatile pattern, allowing releasing for GH twice every six hours in a 24 hour period. Interestingly, in addition to SST effects on other areas of the brain, the gut, and the endocrine and exocrine pancreas, SST is also found in D cells of the gut and δ cells of the endocrine pancreas. GnRH neurons originate in the olfactory bulb and migrate to their final positions in the hypothalamus. Therefore, GnRH neurons tend to be scattered along the migratory path from the olfactory bulb to the hypothalamus. A number of factors stimulate release of GnRH, including norepinephrine, GABA, glutamate, neuropeptide Y, neurotensin, DA, angiotensin II, and interleukin 1 and 2. Gonadal hormones exert an inhibitory and, at certain times in females, a stimulatory effect on GnRH release as well (discussed below). TRH neurons also migrate to their final positions, originating first in the optic recess before migrating to the preoptic area and

lateral hypothalamic region. TRH neurons are noradrenergic and excess glucocorticoids can inhibit release of TRH. The presence of glucocorticoid receptors on TRH neurons in the paraventricular region further substantiates this. Additionally, thyroid hormones are thought to regulate TRH release via transcription and translation. As mentioned earlier, unlike many of the other pituitary hormones, PRL does have a known stimulatory hypothalamic hormone but instead constant release of dopamine creates a tonic inhibition of PRL release. The suckling action of breastfeeding inhibits DA release, thus allowing PRL to be released. The more PRL that is released, the more DA release is inhibited as well. DA has a similar action on release of melanocortin stimulating hormone (MSH) from the intermediate lobe of the pituitary gland.

Although not considered a crucial part of hypothalamic development, *Pax6* is expressed in parts of the developing hypothalamus and important for patterning and establishing boundaries in hypothalamic regions even where it is not expressed. At E10.5, *Pax6* expression in the supraoptic/paraventricular region, anterior hypothalamic area, and posterior preoptic area plays a significant role in patterning of these hypothalamic regions, becoming restricted to only the paraventricular region by E14.5 (13). Furthermore, the complimentary expression of *Pax6* and *Dlx1* defines the boundaries between the posterior and anterior preoptic areas, the posterior preoptic area and suprachiasmatic area, and the supraoptic paraventricular area and hypothalamic cell cord (14). In the complete absence of *Pax6*, these regions become hypoplastic while hypothalamic regions where *Pax6* is not expressed become broader, likely as a result of broadened *Dlx1* expression and loss of defined boundaries within the hypothalamus.

Since null *Pax6* mutants do not survive past the first day of birth, the effects on the hypothalamic endocrine hormones of these regions have not been studied (15). To date, no studies have looked at the affects of a haploinsufficiency in *Pax6* on hypothalamic hormones.

Still, a heterozygous mutation in *Pax6* has been shown to affect a number of tissues where *Pax6* is expressed, both in human and mice. Thus, studies to see if there is a change in hypothalamic hormones in *Pax6* deficient mice and/or Aniridic patients would be worthwhile.

Pituitary Gland

The pituitary gland is considered the executor of the endocrine system, sitting at the base of the brain underneath the hypothalamus, taking its cues from the hypothalamus and responding by sending out the corresponding hormone. The pituitary gland is made up of three different lobes: the posterior lobe, an extension of neural ectoderm from the ventral diencephalon, and the anterior and intermediate lobes, which are derived from the oral roof ectoderm. The hormones of the posterior lobe, oxytocin (OT) and arganine vasopressin (AVP), have cell bodies that are found in the hypothalamus and nerve endings that end in the posterior lobe. The intermediate lobe secretes melanocortin stimulating hormone (MSH) while the anterior lobe secretes the corticotrope adrenocorticotropin hormone (ACTH), the somatotrope growth hormone (GH), the lactotrope prolactin (PRL), the thyrotrope thyroid stimulating hormone (TSH), and finally two gonadotropes, leutenizing hormone (LH) and follicle stimulating hormone (FSH). The anterior lobe hormone producing cells develop in a dorsal to ventral pattern, with corticotropes being most dorsal, somatotropes, lactotropes, and thyrotropes following next, dorsal to ventral, and gonadotropes being most ventral. The six hormones released from their particular cell type participate in the regulation of stress response, growth and metabolism, milk production, and reproduction.

As described extensively by Rizzoti and Lovell-Badge, development of pituitary gland begins around E7.5 in mice with a thickening of the hypophyseal placode at the midline of the anterior neural ridge (16). 24 hours later, at E8.5, due to rapid growth of the neural tube and its

bending at the cephalic region, the hypophyseal placode is positioned directly underneath the forebrain within the oral roof ectoderm (Figure 1.3). By E9.0, the hypophyseal placode invaginates dorsally towards the infundibular region of the hypothalamus, forming a rudimentary Rathke's pouch. This placement of the placode under the hypothalamus allows direct contact with the infundibulum. This direct contact with this neural ectodermal region is necessary for growth of early pouch and gland as evidenced by the complete absence of the pituitary gland when the region containing the infundibulum is genetically ablated (17). At E10.5, the fully invaginated pouch allows for the infundibulum to begin to evaginate ventrally towards the pouch (Figure 1.3). This contact with the infundibulum is required for proper development and hormone cell differentiation of the Rathke's pouch. Two days later, at E12.5, the Rathke's pouch is completely separated from the underlying oral roof ectoderm, and the first hormone producing cell types are beginning to differentiate.

Pituitary gland development involves a number of different signals that are each required for proper development and cell differentiation. This begins with *Bmp4* expression in the infundibulum at E8.5, just as the hypophyseal placode comes into contact with it (18). Indeed, if *Bmp4* is not expressed, the Rathke's pouch will not develop (19). Additionally, *Bmp4* helps to maintain expression of *Isl1*, which is required for precursor proliferation, is expressed in the early Rathke's pouch. Additionally, *Fgf8*, *Fgf10*, and *Fgf18* are also expressed in the infundibulum by E9.5 and are important for proliferation and emergence of dorsal pouch cell types (18,20). *Fgf8*, for example, is required proliferation of POMC precursors, but its continued expression prevents terminal differentiation of ACTH and must first be down-regulated for terminal ACTH differentiation around E14.5. Ventrally, *Bmp2* expression begins around E10.5 between the pouch and underlying oral ectoderm, becoming expressed throughout the pouch by

E12 (18). *Bmp2* expression is thought to be important for ventral precursor proliferation and cell fate, although, much like *Fgf8*, its down-regulation is required for final differentiation of ventral cell types. Although *Shh* expression is excluded from Rathke's pouch, it is expressed throughout the oral ectoderm where it is thought to be important for induction of *Bmp2* expression, which, as mentioned earlier, is required for ventral cell type specification and expansion (21).

The signaling molecules discussed above, in addition to members of the *Wnt* family, activate and/or repress a number of genes and transcription factors that are also required for development of the Rathke's pouch and differentiation and proliferation of cell types. Three members of the *Pitx* family, *Pitx1*, *Pitx2*, and *Pitx3* are expressed in the developing and adult pituitary gland. *Pitx1* activates transcription of a number of other genes and transcription factors while *Pitx2* expression is critical for development of pituitary primordia and, with the exception of ACTH-producing cells, critical for the final differentiation of every cell type (22–24). Not much is known about *Pitx3* except that it is expressed in all pituitary cell types (25,26). Members of the LIM homeodomain family are critical for pituitary gland development as well. *Isl1* is required for proliferation of pituitary precursors, as evidenced by the finding of only a rudimentary pouch in *Isl1* null embryos (18,19). *Lhx4* is also required for early development and expansion of Rathke's pouch, though not essential for subsequent differentiation of most cell types in the pituitary (27). *Lhx3* is expressed in the developing Rathke's pouch and maintained in the adult gland. It also activates transcription of other transcription factor genes and hormone genes, such as Pit1 (discussed below) and *Prl*, TSH, and α-GSU (28–30). The requirement of *Lhx3* in the developing pituitary gland is further confirmed by the lack of anterior and posterior lobes in null mutants.

Six3 and *Six6* are also expressed in the embryonic pituitary, thought to be important for proliferation of intermediate and dorsal precursor cell proliferation. *Six3* is first detected in the anterior border of the neural plate and in the absence of *Six6*, pituitaries are hypoplastic (31,32). The importance of *Pit1* for development of somatotropes, lactotropes, and thyrotropes was first made evident through the Snell and Jackson strains of dwarf mice. The actions of *Pit1* on the afore mentioned cell types works by activating some genes while repressing others - such as repressing gonadotrope lineage to give thyrotrope lineage (33,34). *Prop1* was first thought to be required for activation of *Pit1* gene, but a recent study has implicated it as necessary for specification of all pituitary hormone cell types (35). Gonadotrope lineage cell types require steroidogenic factor 1 (*SF1*) for their development – as evidenced by *SF1* mutant mice that had an absent of gonadotropes and agenesis of gonads and adrenal glands (36,37). *Tpit*, expressed in POMC producing cells of the anterior and intermediate lobes, activates their expression by synergizing with *Pitx1* (38,39). Although *NeuroD1* is required for initial corticotrope specification, it is *Tpit*, along with *Pitx1*, that is required for its final differentiation. Indeed, mutant mice with inactivated *Tpit* did not grow any melantropes in the intermediate lobe but instead were populated with gonadotropes (40).

Final differentiation of anterior pituitary hormone cell type begins at E12.5 and is complete a few days before birth, at E17.5 (41). Both corticotropes and intermediate lobe melanotropes are derived from the POMC gene (proopiomelanocortin). For corticotropes, *Tpit* synergizes with *Pitx1* to activate POMC regulatory elements (22,39). *Pitx1* also interacts with *NeuroD1* to upregulate POMC expression, which is then cleaved at four sites to give rise to ACTH in the anterior lobe (42). The final adult population of thyrotropes differentiates next at E14.5. *Gata2* is expressed in all ventral cell types (including the two gonadotropes) as they

share a common lineage (43). Although *Gata2* expression at the most ventral location represses *Pit1* expression, at the more intermediate location, *Gata2* is not expressed strongly enough to inhibit *Pit1* expression and the two both act to activate transcription of TSH (33). Somatotrope and lactotropes undergo terminal differentiation next at E15.5. The presence of *Pit1* is one of the main defining characteristics of somatotropes, along with the presence of *Egr1*, thyroid hormone receptor (T3R), and *Otx1* (44,45). Lactotropes, as mentioned earlier, also are defined by the expression of *Pit1* as well as estrogen receptors to activate its transcription (46). Gonadotropes are the last to undergo terminal differentiation at E16.5 for LH and E17.5 for FSH. In addition to SF1, as mentioned earlier, gonadotrope lineages also require *Pitx1* and *Egr1* for differentiation as well(47–49).

Upon release, ACTH acts on the adrenal glands, which sit on top of the kidneys, to stimulate synthesis and release of glucocorticoids. Glucocorticoids are released as part of the stress system and are also important in carbohydrate metabolism since glucocorticoids also affect glucose and cellular metabolism. Glucocorticoids are also involved in a negative feedback regulation by inhibiting secretion of CRH and/or ACTH from the hypothalamus and pituitary gland, respectively, when there is enough circulating through the body. An excess of circulating glucocorticoids leads to Cushing's disease in humans, which can affect fat distribution, menstrual cycle in women, heart rate, weight gain and abnormal glucose among a host of other symptoms. Treatment for Cushing's disease depends on the cause for the excess hormone and can involve drugs that inhibit the action of glucocorticoids or some form of surgery or chemotherapy in severe cases, as with pituitary adenomas. A deficit in circulating glucocorticoids, clinically known as Addison's disease, can result in chronic fatigue and muscle weakness, low blood pressure, low blood sugar, menstrual irregularities, and, in some cases,

hyperpigmentation. Treatment usually involves exogenous glucocorticoid therapy. GH has a crucial role in growth, particularly in children and adolescents, and metabolism. It acts on the liver, which produces and releases insulin like growth factors (IGFs) in response, and the bone. It also acts on muscle tissue to enhance protein incorporation and stimulates growth of a number of additional tissues and organs. During childhood, GH levels are constant, reaching their peak during the maximal growth period of adolescence. Thereafter, GH release is a bit more cyclical, released approximately two times during a 6-hour period, as a result of the concerted action of GHRH and SST from the hypothalamus. GH also regulates GHRH synthesis and release through a negative feedback loop to regulate GH homeostasis. A deficit in GH can result in short stature while an excess can result in gigantism if the excess occurs during childhood and acromegaly if the excess is during adulthood. Symptoms of acromegaly include thickening of the skin and subcutaneous tissue and enlarged visceral organs, including the heart, liver, lungs, and kidneys. Additionally, an increase in metabolism and hyperglycemia are often symptomatic of acromegaly as well. A slow-release exogenous somatostatin therapy is usually used to treat gigantism and acromegaly while GH replacement therapy is used to treat short stature.

The actions of prolactin on mammary glands relies on several additional hormones, including estrogens, insulin, glucocorticoids, progesterone, and GH. Estrogens in particular stimulate transcription and synthesis of PRL. PRL levels increase after puberty and during pregnancy, but fall back to basal levels within three weeks after pregnancy if no breastfeeding occurs. In females whom are not pregnant, PRL helps to regulate the menstrual cycle. In males, PRL has been suggested to affect accessory sex organs and fertility (50,51).

TSH acts on the thyroid gland to stimulate release of the thyroid hormones thyroxine (T_4) and triiodothyronine (T_3). Thyroid hormones play a significant role in early nervous system

development and differentiation, regulating basal metabolic rate, bone growth and maturation (both directly and indirectly through its permissive action on GH). They also affect the reproductive system, cardiovascular system, lipid and carbohydrate metabolism (thus regulating body weight) and the fully developed brain. It also affects glucose metabolism by increasing the rate of intestinal glucose absorption as well as enzyme synthesis in a number of tissues. While T_4 is much more abundant that T_3, T_3 is the more biologically active form as it is more potent than T_4 for most physiological responses. Still, T_4 is thought to be the hormone that regulates transcription of TRH in the paraventricular region of the hypothalamus and is exceptionally high in newborn cord blood, suggesting it has a direct functional role during development. Much like glucocorticoids and GH, thyroid hormones also inhibit release of TRH and TSH through a negative feedback loop, thus maintaining a balance of circulating thyroid hormones. Excess thyroid hormones, or hyperthyroidism, results in elevated metabolism, increased heart rate, weight loss or inability to gain weight, menstrual irregularities, and often leads to goiters (a swelling of the thyroid gland) due to unchecked thyroid hormone production. Antithyroid drugs are used to treat non-cancerous cases of hyperthoidism while in cases of cancerous growths, surgery and/or radiation is the best mode of treatment. Hypothyroidism, or a deficit of circulating thyroid hormones, results in decreased or low metabolism, decreased heart rate, coarse, dry skin, lethargy, weight gain, and menstrual irregularities. This is usually treated with oral administration of levothyroxine, a synthetic form of T_4.

The actions of LH and FSH differ for male and female, although both act on the gonads to produce gonadal steroid hormones. In males, pituitary gonadotropes act on the Leydig and Sertoli cells within the testes to control synthesis of testosterone and spermatogenesis. Specifically, LH stimulates testosterone secretion from the Leydig cells while FSH is required

for development and production of sperm within the Sertoli cells. Testosterone is further required for a number of processes, particularly during puberty in males. This includes enhanced growth of skeletal muscle (masculine body growth), bones, voice change by its action on vocal cords, penile and scrotal growth, libido, hair growth, and sebaceous gland growth and sebum production. It also acts to increase the content of accessory reproductive glands in males, such as the prostate and seminal vesicles. In females, FSH acts on granulosa cells within the ovaries to produce estrogen and LH acts on thecal cells to produce progesterone. Estrogens are required for a host of physiological properties during development and in adults, including proper growth and development of the vagina, uterus, and oviducts. It also maintains libido, sexual behavior, and facilitates maternal behavior. It also increases oxytocin and prolactin production in the pituitary gland, is required for maturation of the ovum, and is responsible for fat distribution in the adult female and zygote maturation in the pregnant female. Progesterone is considered the ovarian hormone of pregnancy and acts to prepare the reproductive tract for zygote implantation and maintaining pregnancy upon implantation. Gonadal hormones have an inhibitory effect on GnRH release from the hypothalamus.

OT from the posterior lobe controls milk release after childbirth (not to be confused with lactogenesis of PRL of the anterior lobe). During suckling, sensory nerve endings located around the areolas and nipples stimulate OT release. Although its main mode of action is milk release, it also controls uterine wall contractions during childbirth, has a role in maternal behavior and plays a role in male and female mating behavior (52). Arganine vasopressin (AVP), also known as antidiuretic hormone (ADH), regulates blood plasma osmolarity by inducing contraction or relaxation of smooth muscle, resulting in movement of water or sodium across the distal tube of the kidneys (in mammals). When blood plasma levels becomes too thin,

circulating AVP levels decrease, resulting in excretion of heavily diluted urine. Conversely, when blood plasma is too thick, it stimulates AVP release, stimulating water retention and excretion of smaller volumes of less dilute urine. Although rare, if there is a failure of AVP synthesis or release due to injury to the posterior lobe (or the region of the hypothalamus where AVP cell bodies are located), it results in an inability of the kidney to conserve water, or pituitary diabetes insipidus. This can be treated by AVP hormone replacement therapy. A condition known as the "syndrome of inappropriate vasopressin secretion" results when there is sustained, continual AVP release without regard to blood plasma volume. Ultimately this results in an inability of the body to excrete properly diluted urine and excess retention of water. Finally, the intermediate lobe controls melanin pigmentation through α-MSH release, which acts on melanocytes found in the skin.

The actions of *Pax6* on the pituitary gland are not extensively studied. In the developing mouse pituitary gland it was found to be expressed beginning around E8.5 and maintained through E18.5. In determining the role of *Pax6* in anterior lobe development, Kioussi et al found that it is expressed in the developing Rathke's pouch, restricted dorsally until about E12.5. The expansion of ventral thyrotropes and gonadotropes and decreased dorsal-intermediate in the complete absence of *Pax6* indicates a role for it in dorsal-ventral patterning of the developing pituitary gland (Figure 1.4). Still, no studies to date have shown a mechanistic role for *Pax6* in pituitary hormone cell type development. *Pax6* expression in development of the pituitary gland begins in the oral ectoderm from which the Rathke's pouch invaginates and, as will be discussed later, continues in the mature gland through adulthood. Further studies looking into the mechanism of *Pax6* actions on the pituitary will be beneficial in determining its role in pituitary gland development and function.

Pancreas

Although most of the endocrine system involves communication between the brain, pituitary gland, and the organs it stimulates to release hormones, glucose homeostasis involves the careful balance of hormones released from the endocrine pancreas. The pancreas is mostly comprised of acinar cells of exocrine function, which secrete digestive enzymes, a ductal system through which acinar enzymes are transported to other digestive organs, and an endocrine portion, known as islets of Langerhans, that is comprised of the hormone-producing cells involved in glucose homeostasis. Islets are composed of five different cell types which secrete its respective hormone, insulin producing β-cells – which make up the majority of cells in islets, glucagon-producing α-cells, somatostatin producing δ-cells, pancreatic polypeptide producing γ-cells, and ghrelin producing ε-cells. In mice, the β-cells form the inner core of islets with the other four cell types surrounding them (Figure 1.5).

Comprised of epithelial tissue, the pancreas develops as two lobes that arise as thickenings along the dorsal and ventral portions of the foregut, around E9.0 in mice (53). During development, the dorsal lobe begins to bud first, followed by the ventral bud shortly after. These initial buds form as a result of interactions between mesenchymal and epithelial cells, inducing bud formation and branching, with signals from mesenchymal cells directing endocrine or exocrine fate and rotation of the gut brings the two lobes closer together and they fuse by E16-17 (54). Endocrine cells can be found within the early stages of bud development, forming islets during the final few days of gestation.

All islet cells originate from a common pluripotent progenitor pool, undergoing several differentiation steps to form each endocrine cell type (55,56). These differentiation steps involve the expression of a number of organ and cell-specific transcription factors. *Pdx1* and *Ptf1a* are

two transcription factors that are required for pancreas organ development and expressed in the early pancreas (57–59). While *Ptf1a* becomes restricted to acinar exocrine precursor cells by E13.5, *Pdx1* is expressed in all pancreas cell types, including acinar, ductal, and islet cells. However, *Ngn3* is specifically required for endocrine cell fate in the developing mouse pancreas, as it is an early marker of islet precursors (60). *Ngn3* expression in cells of endocrine fate significantly increases during mid-embryogenesis, activating a number of transcription factors before its expression declines toward birth when final markers begin to be expressed (61). These transcription factors include *Isl1*, *NeuroD1*, and *Insm1/IA1*, which are expressed in all endocrine cell types (62–65). After expression of these three important transcription factors, the different cell types emerge as a result of expression of cell type specific transcription factors. These include *Arx* for α-cell formation, *Nkx2.2* for γ-cell formation, *Pax4* for δ-cell formation, and *Nkx2.2*, *Pax4*, and *Nkx6.1* for β-cell formation (66–71). Still, the transcription factor *Pax6*, which is downstream of *Ngn3* has also been shown to be required for development of 4 of the 5 endocrine pancreas cell types.

In *Pax6* homozygous null mutant mice, there is a complete absence of α-cells, a marked reduction in the number of β, δ, and γ cell types, and an increase in ε-cells (Sander et al. 1997; Heller et al. 2005). Furthermore, *Pax6* expression in the pancreas begins at E9.0 in the region of the primitive gut that will give rise to the pancreas (Sander et al. 1997). In addition to being expressed in α-cells at E10.5, concurrent with α-cell appearance, *Pax6* has been shown to regulate expression of other genes that are involved in α-cell differentiation, β-cell maturity and function, and glucagon and insulin biosynthesis (75–77). While *Pax6* expression is restricted early in islet development, it is expressed throughout the pancreas towards the end of the development and in mature islets of Langerhans.

It has been suggested that α, β, δ, and γ cell types have a common DNA sequence through which *Pax6* has a direct role in regulating transcription. Additionally, in $Pax6^{SeyNeu}$ null mutant mice, there is a decrease in levels glucagon and insulin levels, indicating a role for *Pax6* glucagon and insulin synthesis as well. This was validated by the finding that *Pax6* directly regulates transcription of the proglucagon gene as well as PC2, the prohormone convertase that processes it to glucagon in the islet (78,79). Similarly, *Pax6* is also involved in insulin gene transcription and insulin prohormone processing and secretion through regulating transcription of PC1/3 (77,80). Its role in regulation of PC1/3 transcription and synthesis was further verified in the case of haplo-insufficiency with human patients with Aniridia (81). Indeed, although haploinsufficient mice showed no difference in islet morphology, Aniridic patients have been shown to have severe glucose intolerance as a result of defective insulin secretion, as a result of PC1/3 deficiency (81–83). Finally, expression of the glucose transporter Glut2 was also found to be reduced in the pancreas of *Pax6* deficient mice (84).

Glucose homeostasis involves a coordinating relationship between α and β cells to maintain a balance of glucose production and utilization. If glucose levels are not properly regulated, it can result in blood glucose levels remaining abnormally high, otherwise known as diabetes. If insulin synthesis or release is impaired, it can result in excess glucose production by the liver, lipid and protein breakdown, and death, if left untreated. As mentioned previously, β-cells produce insulin, which directly lowers blood glucose levels by stimulating uptake of glucose into tissues. Insulin is released first as an inactive prohormone and must be enzymatically cleaved to produce the active form of the hormone. For proinsulin, this is achieved through the prohormone convertase PC1/3. Levels of insulin circulating through the blood are monitored by insulin receptors known as glucose transporters, notably GLUT2 in the

pancreas and liver and GLUT4 in adipose and muscle tissue. Elevated levels of circulating blood glucose leads to release of insulin from β-cells. In the liver, it activates glycogen synthetase to push glucose to glycogen formation. In adipose tissue, it acts on fat cells to facilitate release of free fatty acids from chylomicrons, which are then transported into fat cells where they combine with glycerol to form triglycerides. Finally, it acts on muscle cells to activate transport of glucose and amino acids into muscle cells, ultimately resulting in enhanced protein synthesis.

Glucagon is also produced in an inactive prohormone form, proglucagon, that undergoes post-translational modifications to produce glucagon. In the pancreas, this is done through the prohormone convertase PC2. Interestingly, it is processed differently in intestinal enteroendocrine cells to produce GLP-1 and GLP-2, which will be discussed later. When blood glucose levels are low, glucagon acts on the liver and adipose tissue to elevate blood glucose levels. It acts on the liver to activate glycogenolysis and gluconeogenesis and acts on fat tissue to break down lipids into free fatty acids and glycerol. The remaining cell types of the endocrine pancreas are less plentiful than β and α-cells. δ-cells produce somatostatin and are also thought to have a paracrine role in insulin and glucagon production by inhibiting their secretion. Similarly, pancreatic polypeptide released from γ-cells acts on both the endocrine and exocrine, its secretion being particularly increased after a high protein meal and/or during periods of exercise, fasting, and hypoglycemia.

Upon ingestion of exogenous carbohydrates, glucose levels in the blood spike. This inhibits glucagon release while at the same time stimulating insulin release. If there is no insulin present, such as in cases of diabetes, glucose homeostasis is severely compromised. Insulin-dependent diabetes mellitus results from auto-antibodies that attack and prevent proper functioning of β-cells. Non-insulin dependent diabetes mellitus results from decreased

sensitivity of target tissues to the actions of insulin and/or inability of β-cells to secrete insulin due to overwhelmed or continued hyperglycemic state. A sustained hyperglycemic state reverses all effects of insulin on bodily tissues, resulting in their inability to take up glucose. The liver no longer synthesizes glycogen, resulting in the loss of glucose from hepatocytes. Since there is increased circulating glucose, there is less glucose available for glycolysis and ATP production. Furthermore, the excess circulating glucose leads to excretion of large amounts of urine, causing dehydration and intense thirst. Additionally, the inability of adipose tissue to take up glucose results in increased release of free fatty acids and glycerol, which the liver uses for gluconeogenesis, further exacerbating the hyperglycemic state. Protein catabolism also results due to inability of glucose and amino acids to enter muscle cells.

Since hyperglycemia has such catastrophic effects, exogenous insulin is used to treat both forms of diabetes. Much research is being done to produce new β-cells in the pancreas, and although there is some promising research, to date, none have yet resulted in the production of new insulin-producing cells.

For this dissertation we were particularly interested in how a haploinsufficiency in *Pax6* affects the various part of the endocrine system. Although the effects in the small intestine were explored and its role in the hypothalamus hypothesized, we chose to focus on its role in the pituitary gland. The development and organization of the pituitary has been well described and pituitary hormones are easily accessible and understood. Furthermore, several studies have previously addressed changes in pituitary hormones in human patients with Aniridia (81,85,86). As stated earlier, in a previous study, Kioussi, et al found that in the complete absence of *Pax6*, there is a loss of two dorsal cell types and an expansion of ventral cell types (87). Therefore, we chose to investigate the possibility that even a haploinsufficiency in *Pax6* may change pituitary

hormone-producing cell numbers and, thus, hormone levels. Any potential change in hormone levels found in mice, if replicated in human patients, could explain a host of symptoms in patients.

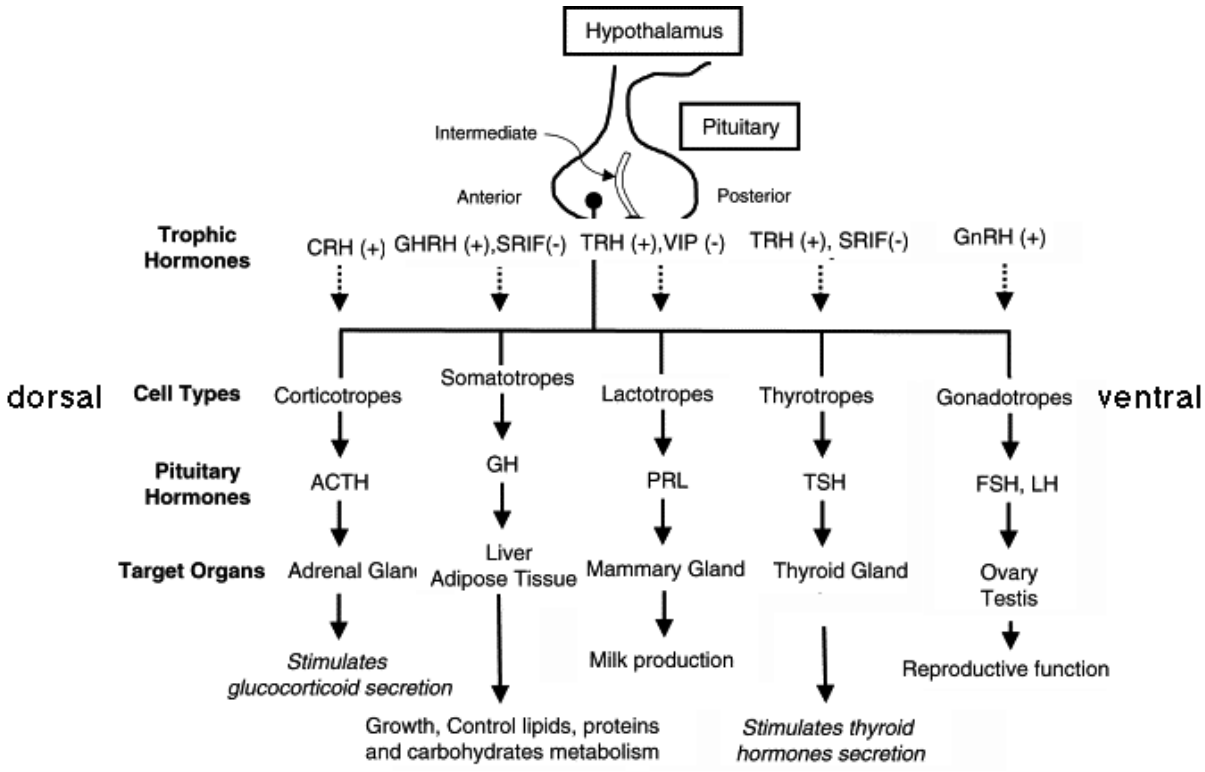
Hypothalamus
Pituitary
Intermediate
Anterior
Posterior
Trophic Hormones
CRH (+)
GHRH (+),SRIF(-)
TRH (+),VIP (-)
TRH (+), SRIF(-)
GnRH (+)
dorsal
Cell Types
Corticotropes
Somatotropes
Lactotropes
Thyrotropes
Gonadotropes
ventral
Pituitary Hormones
ACTH
GH
PRL
TSH
FSH, LH
Target Organs
Adrenal Gland
Liver
Adipose Tissue
Mammary Gland
Thyroid Gland
Ovary
Testis
Stimulates glucocorticoid secretion
Milk production
Reproductive function
Growth, Control lipids, proteins and carbohydrates metabolism
Stimulates thyroid hormones secretion

Figure 1.1

Figure 1.1 Hypothalamic hormones are the master regulator of the endocrine system
Hypothalamic hormones, named for the pituitary hormone that they stimulate release of, act directly on the pituitary gland. Upon stimulation, the pituitary gland then acts directly on its particular organs and/ or glands to stimulate release of its corresponding hormone. These hormones then exerts their effects on the body homeostasis, including growth, metabolism, reproduction, and stress.
Abbreviations: coricotropin releasing hormone (CRH), adrenocorticotropin hormone (ACTH), growth hormone releasing hormone (GHRH), somatotropin release inhibiting factor (SRIF, also known as somatostatin), growth hormone (GH), prolactin (PRL), thyroid releasing hormone (TRH), thyroid stimulating hormone (TSH), gonadotropin releasing hormone (GnRH), follicle stimulating hormone (FSH), leutenizing hormone (LH).

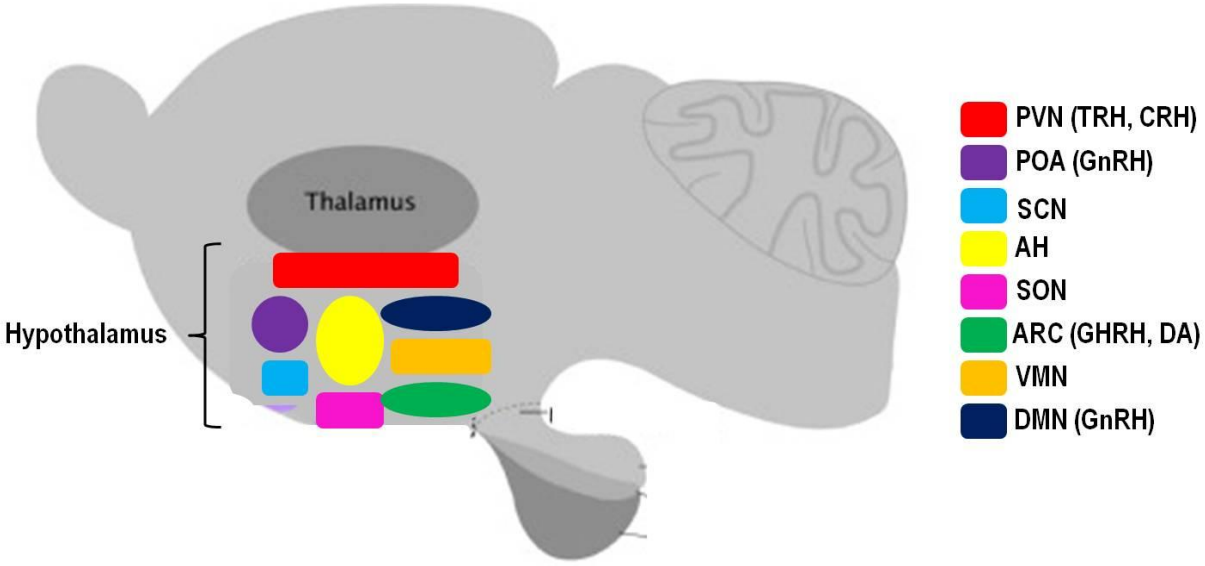
Thalamus
Hypothalamus
PVN (TRH, CRH)
POA (GnRH)
SCN
AH
SON
ARC (GHRH, DA)
VMN
DMN (GnRH)

Figure 1.2

Figure 1.2 Location of Hypothalamic Nuclei within the hypothalamus
The hypothalamus is found directly below the thalamus and consists of clusters of nuclei that surround the third ventricle and sits at the base of the diencephalon. These cluster regions include the Suprachiasmatic Nucleus (SCN), supraoptic nuclei (SON), preoptic area (POA), anterior hypothalamus (AH), paraventricular nucleus (PVN), arcuate nucleus (ARC), dorsomedial nucleus (DMN), and ventromedial nucleus (VMN). The hormone containing neurons of the hypothalamus are located at various locations within these nuclei clusters. TRH and CRH neurons are located in the PVN. In rodents, GnRH neurons are found in the POA but in humans they are located in the DMN. GHRH and hypothalamic DA neurons are located in the ARC. AVP and OCT cell bodies are found in the PVN (not shown).

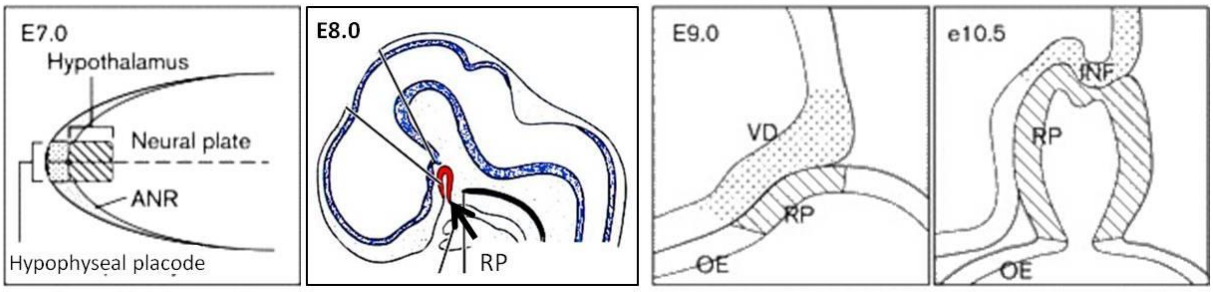
E7.0
Hypothalamus
Neural plate
ANR
Hypophyseal placode
E8.0
RP
E9.0
VD
RP
OE
e10.5
INF
RP
OE

Figure 1.3

Figure 1.3 Development of the Rathke's pouch
Development of the pituitary gland begins with thickening of the hypophyseal placode at E7.0 in mice. Rapid proliferation of the neural tube causes a bending of the embyo at the cephalic region by E8.0, bringing the presumptive Rathke's pouch directly underneath the ventral diencephalon. Beginning at E9.0, the Rathke's pouch develops as an outgrowth of the oral roof ectoderm, which invaginates dorsally. The close proximity of the pouch to the ventral diencephalon is critical for continued development as well as for stimulation of evagination of the infundibulum. By E10.5, the Rathke's pouch has fully invaginated and the infundibulum has begun to evaginate and surround the Rathke's pouch. Abbreviations: anterior neural ridge (ANR), rathke's pouch (RP), ventral diencephalon (VD), oral ectoderm (OE), infundibulum (INF).

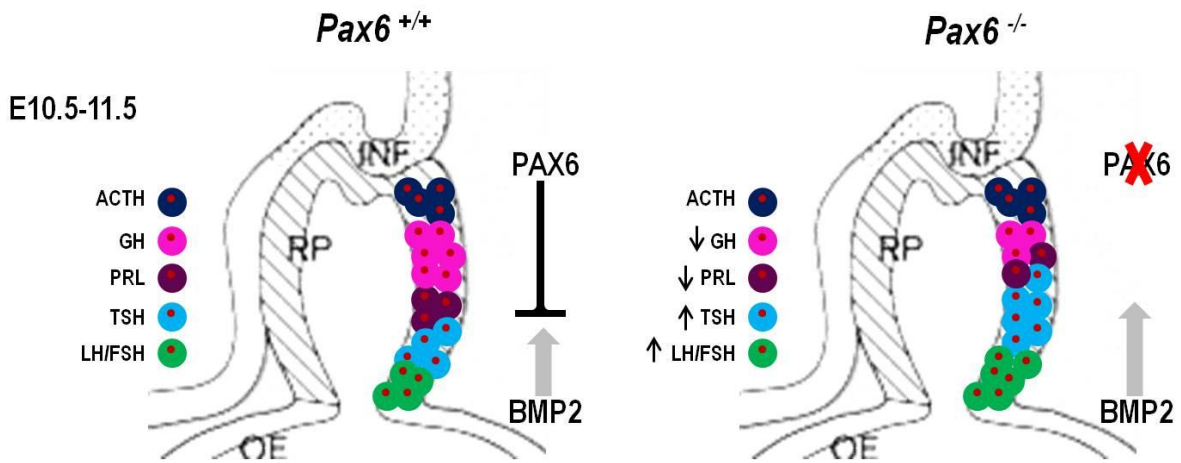

Figure 1.4

Figure 1.4 *Pax6* affects dorsal-ventral patterning of the Rathke's pouch
Cell types of the anterior lobe develop in a dorsal-to-ventral pattern, with corticotropes (ACTH) being dorsal-most, followed by somatotropes (GH) and lactotropes (PRL) in the dorsal-intermediate region, thyrotropes (TSH) in the ventral-intermediate region, and gonadotropes

(LH/FSH) ventral-most. Between E10.5 and E12.5, *Pax6* expression is restricted to the dorsal part of the Rathke's pouch. In the complete absence of *Pax6*, expression of BMP2 expands dorsally, which results in a change in hormone-producing cell types. Although corticotropes are unaffected, there is a reduction in the number of the more dorsal somatotropes and lactotropes cell types and an increase in the number of the ventral cell types, thyrotropes and gonadotropes.

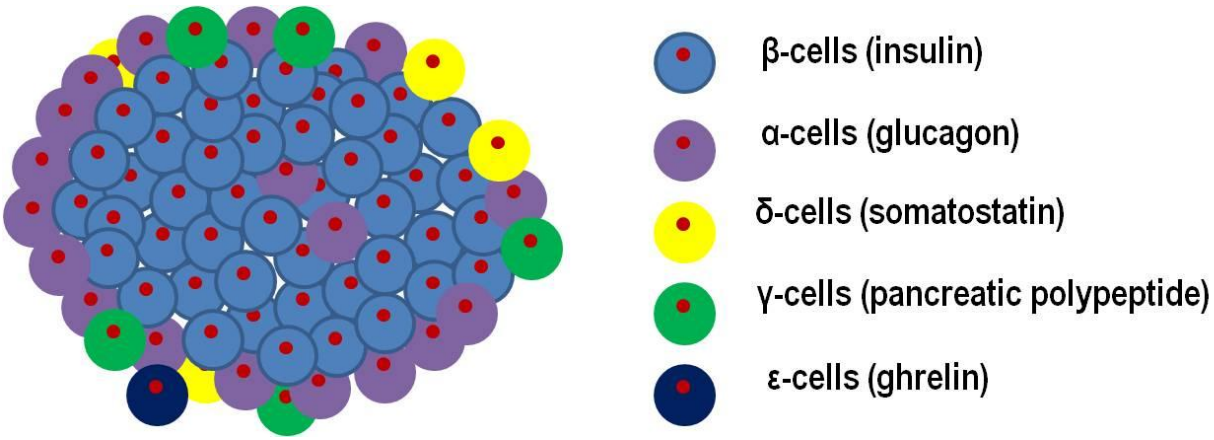
β-cells (insulin)
α-cells (glucagon)
δ-cells (somatostatin)
γ-cells (pancreatic polypeptide)
ε-cells (ghrelin)

Figure 1.5

Figure 1.5 Cell types and distribution in the pancreatic islets of Langerhans
The endocrine pancreas, the islets of Langerhans, is a cluster of endocrine hormone-producing cells within the larger exocrine pancreas. The islets are made up of five cell types that release their respective hormone that are critical for glucose homeostasis as well as some digestive processes. These cell types include the insulin secreting β-cells, glucagon secreting α-cells, somatostatin secreting δ-cells, pancreatic polypeptide secreting γ-cells, and ghrelin secreting ε-cells. The most abundant β-cells are concentrated at the core, surrounded by the other cell types, with α-cells being the next most abundant.

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CHAPTER 2

INCREASED TSH-PRODUCING CELLS IN THE PITUITARY GLAND OF *Pax6* HAPLO-INSUFFICIENT MICE

Submitted to *Endocrinology*. Kenji K. Johnson, Anastasia M. Bobilev, Khan Hekmaytar, and James D. Lauderdale, 4/19/16.

Increased TSH-producing cells in the pituitary gland of Pax6 haplo-insufficient mice

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Abbreviated title: Pax6-dependent changes in adult mouse pituitary

Key words: Magnetic Resonance Imaging, *Small eye* mouse, hormone regulation, endocrine

Word count: (excluding abstract, figure captions, and references): 6413

Numbers of figures and tables: *6 Figures; 1 Antibody table as requested*

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Grants and Fellowships: This work was supported by a grant from the Sharon Stewart Aniridia Research Trust to JDL. KKJ was supported by a graduate fellowship from the Southern Regional

Education Board Doctoral Scholars Program, and AMB was supported by a graduate fellowship from John and Mary Franklin Foundation Neuroimaging Training Program and is an ARCS Scholar. Collection of magnetic resonance imaging data was made possible by the University of Georgia Bioimaging Research Center and NIH Shared Instrumentation grant S10RR023706.

Disclosure Statement: The authors have nothing to disclose. The funders of this research had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Aniridia is a congenital condition characterized by absence of iris and is caused by a semidominant mutation in the transcription factor encoded by the *Pax6* gene. Although ocular phenotypes of this disorder are well characterized, recent studies report that individuals with aniridia have a higher propensity for obesity, infertility, polycystic ovarian disease, and severe eczema compared to their *Pax6*-normal siblings. These symptoms collectively suggest an underlying endocrine disturbance related to haploinsufficient levels of *Pax6*. In mice, during development, *Pax6* expression in the pituitary gland begins at E9.0 in the primordial anterior pituitary gland (Rathke's Pouch). This expression becomes restricted to the dorsal anterior pituitary by E11.5, but is expressed throughout the anterior lobe by E14.5, and remains through adulthood. It is possible that a reduction in *Pax6* could result in a change in pituitary hormone levels or cell numbers, which may explain symptoms experienced by aniridics. Using the *Small eye* mouse model, we find that *Pax6* reduction results in a decrease in GH-producing cells and an increase in TSH-producing cells in neonate mice, with the TSH increase continuing into adulthood. Adult *Pax6* haploinsufficient mice also have an increase in anterior pituitary volume and weigh significantly less than their wild-type littermates. Furthermore, we show that the increase in TSH-producing cells leads to an increase in thyroxin (T_4) in mutant mice, although tri-iodothyronine (T_3) levels remain unchanged. These findings present a new role for *Pax6* in the endocrine system, which serves to refine our current understanding of *Pax6* in endocrine development and maintenance and provides new avenues for investigating endocrine-related symptomatology in aniridia.

Abbreviations

ACTH, adrenocorticotropin hormone; CSF, cerebro-spinal fluid; FSH, follicle stimulating hormone; GH, growth hormone; H&E, hematoxylin and eosin stain; ISH, in situ hybridization; LH, leutenizing hormone; MRI, magnetic resonance imaging; P, pituitary; PRL, prolactin; TSH, thyroid stimulating hormone; V, third ventricle; VBM, voxel-based morphometry

Introduction

Pax6 is a highly conserved transcription factor that is expressed in the developing central nervous system (CNS) where it is required for proper development of tissues such as the eye, forebrain and hindbrain, and spinal cord (1–3). In addition, *Pax6* is also expressed in the pancreas, specifically the islets of Langerhans, the L and K cells of the small and large intestine, and the pituitary gland (3–7). Homozygous mutations in *Pax6* results in lack of eyes and deformed nasal cavity while a heterozygous loss of function mutation in humans causes a condition known as aniridia (8–10).

Aniridia is a rare, congenital disorder that occurs in approximately 1 in 50,000 to 100,000 live births and is mainly characterized by complete or partial absence of the iris (11,12). In addition to the iris, it is well established that it also results in vascularization of the corneas, glaucoma (leading to vision loss), and cataracts (13–15). Although the eye defects associated with *Pax6* and aniridia have been studied, less is known about the systemic effects of a deficiency of *Pax6*. Recently, people with aniridia have self-reported a higher propensity for systemic symptoms including obesity, polycystic ovarian disease, infertility, and severe eczema (16). We hypothesized that these symptoms may be due to a perturbation of the endocrine system, specifically a change in cell numbers within the anterior pituitary gland.

The pituitary gland consists of three distinct parts: the posterior, intermediate, and anterior lobes. While the posterior lobe develops from an evagination of the infundibulum of the hypothalamus, the intermediate and anterior lobes are derived from an invagination of the oral ectoderm into its primordial structure, Rathke's Pouch (17,18). The oral ectoderm begins to thicken and invaginate around E7.0 in mouse development. This invagination continues until the full development of the Rathke's Pouch by E10.5 and is fully separated from the oral roof

ectoderm by E12.5 (19). Upon this separation, the six cell types of the anterior pituitary gland characterized by hormone production begin their differentiation through E18.5 in a dorsal to ventral pattern: Adrenocorticotropin Hormone (ACTH), Growth Hormone (GH), Prolactin (PRL), Thyroid Stimulating Hormone (TSH), Follicle Stimulating Hormone (FSH), and Leutenizing Hormone (LH).

Coincidentally, in addition to its expression in the anterior neural ridge and oral ectoderm beginning at E8.0, *Pax6* is also expressed dorsally in the Rathke's Pouch between E10.5 and E12.5 and throughout the anterior pituitary gland at least through E18.5 in the developing mouse (3). Furthermore, previous studies have shown that in the complete absence of *Pax6*, there is a decrease in the more dorsal cell types GH and PRL and an increase in the ventral cell types TSH and LH (20,21). Although these studies specifically addressed homozygous loss of function of *Pax6*, if these changes in cell numbers are also present in the heterozygous condition, it might explain some of the systemic symptoms experienced by people with aniridia. Further, recent studies have shown an association between *PAX6* mutation in humans and a disruption in pituitary hormone activity (22–24).

Here, we used the mouse model for aniridia known as *Small eye* to investigate a potential change in hormone cell numbers of the anterior pituitary gland in mice heterozygous null for *Pax6*. We show that *Pax6* reduction results in a decrease in GH-producing cells and an increase in TSH-producing cells in neonate mice, with the TSH increase continuing into adulthood. This increase in TSH-producing cells leads to an increase in thyroxin (T_4) in adult mutant mice, although tri-iodothyronine (T_3) levels remain unchanged. These data support the hypothesis that *Pax6* plays a role in the adult endocrine system.

Materials and Methods

Animals

The mice used for this study were maintained as a *Pax6*$^{Sey\text{-}Neu/+}$ colony on a majority CD1 genetic background. Wild-type (*Pax6*$^{+/+}$) littermates were used as controls. The genotype of each animal was determined by PCR as previously described (25). Both males and females were used. All experiments involving mice were conducted in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were performed with approval and oversight of the University of Georgia Institutional Animal Care and Use Committee.

Histology and immunohistochemistry

To rapidly and uniformly preserve brain tissue after euthanization, adult mice were perfused with 1x phosphate-buffered saline (PBS) and 4% paraformaldehyde/PBS (PFA). Tissues obtained from neonatal (P0) animals and dissected adult pituitary glands were preserved by immersion in 4% PFA at 4°C overnight, rinsed in 1x PBS, and then dehydrated stepwise through a graded series of 50, 70, 90, 96 and 100% ethanol, equilibrated with xylene, and embedded in paraffin using a Tissue Tek apparatus (Miles, Ekhart, USA). Serial sections were cut using a rotary microtome at 8 µm, mounted onto slides (Superfrost/Plus; Fisher Scientific, Pittsburg, PA), and dried at 37°C overnight. Tissue sections were deparaffinized by two rinses in xylene followed by rehydration in decreasing concentrations of ethanol, with a final rinse in tap water. Sections used for histology were stained with Mayer's hematoxylin (Sigma) and eosin solution.

Sections used for indirect immunofluorescence were bleached by incubation in aqueous 3% hydrogen peroxide for 10-20 minutes, and subjected to citrate buffer antigen retrieval. Briefly tissue sections were placed into a boiling sodium citrate solution (10mM Citric Acid,

0.05% Tween 20, pH 6.0) for 30 minutes, removed, and allowed to cool for 30 minutes at room temperature. Sections were then rinsed in PBS and blocked for 30 minutes in a solution of 1% bovine serum albumin (BSA; Fisher Scientific, catalog BP1600-100), 5% normal donkey serum (Sigma-Aldrich, catalog D9663) in PBS. Overnight primary antibody incubation was performed in blocking solution at 4°C in a humidified chamber. Unless otherwise stated, primary antibodies were obtained from the National Hormone and Peptide Program (NHPP, Harbor-UCLA Medical Center, Torrance, CA). Primary antibody identities and dilutions were as follows: rabbit anti-ACTH (AFP156102789Rb, 1:100), rabbit anti-GH (AFP5641801Rb, 1:1000), rabbit anti-TSH (AFP1274789Rb, 1:100), and rabbit anti-LH (AFP571292393Rb, 1:100). After removal of the primary antibody, tissues were rinsed three times with PBS for 5 minutes each, blocked for 10 minutes, and incubated for 30 minutes at room temperature with a 1:1000 dilution of donkey anti-rabbit IgG (H+L) secondary antibody conjugated to Alexa Fluor® 647 (ThermoFisher-Invitrogen, catalog A-31573) in blocking solution. After removal of the secondary antibody, the tissue sections were rinsed several times with PBS, the nuclei were labeled with a 1:10,000 dilution of DAPI (4',6-Diamidino-2-phenylindole; Sigma, D9542) in PBS, and then coverslipped with EMS-Fluorogel.

Imaging of immunolabeled sections

Specific signals were visualized using either standard fluorescence microscopy using a Zeiss Axio Imager.D2 or laser scanning confocal microscopy using a Zeiss LSM 510 Meta Confocal Microscope and images acquired using Zeiss image acquisition software. Between 3 and 5 pituitaries were analyzed for each stage and genotype; representative images are shown. N-values for each experiment are provided in the text and figure legends.

Cell counts

For cell counts made of the pituitary glands from neonates, the pituitary glands from *Pax6*$^{+/+}$, *Pax6*$^{+/-}$ and *Pax6*$^{-/-}$ mice at P0 were serially sectioned in their entirety and immunolabeled for ACTH, GH, TSH and LH and counter stained with DAPI as described above. For each of these hormone-producing cell types, the total numbers of specifically immunolabeled cells were counted in every other section and the numbers for each pituitary totaled. The average number and standard deviation (SD) of immunolabeled cells per genotype were determined using three or four animals per group. Statistical comparison between genotypes was performed by analysis of variance (ANOVA).

RNA *in situ* hybridization

RNA *in situ* hybridization was performed as previously described (26), with the exception that the proteinase K digestion was omitted, on tissue sections prepared from 7 months old adult wild-type mice. Sense and antisense digoxigenin-labeled RNA probes were prepared from a BglII-digest of the pMPX2-1 mouse *Pax6* cDNA clone (25) using a DIG RNA labeling kit (Roche). Hybridization and stringent posthybridization wash steps were performed at 65°C.

RT-PCR

Total RNA from whole eyes, pituitary gland, lung and heart from a 9 months old wild-type adult was prepared using TRIzol reagent (ThermoFisher-Invitrogen , catalog 15596026) following the manufacturer's recommended conditions. Total RNA was then treated with the Turbo DNA-freeTM Kit (ThermoFisher-Ambion, catalog AM1907) following the standard protocol to remove potential DNA contamination. The treated RNA was then reverse transcribed (SuperScript Double-Stranded cDNA Synthesis Kit), and the resulting cDNA was amplified by PCR using a mouse *Pax6* primer pair designed by PrimerBank (27–30). This primer pair

(PrimerBank ID: 7305369a1) generates a 285 bp amplicon spanning exons 5a - 7. PCR conditions as previously described (31).

Western Blot

Western analysis was performed on protein lysates prepared from whole eyes, pituitary glands, lung and heart dissected from adults (7 months old). Lysates were prepared by homogenization of tissues in RIPA buffer (10mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.1% SDS, 10% glycerol, 1mM PMSF, 1mM EGTA) in ice. Homogenized samples were centrifuged at 13,000 rpm for 5 minutes at 4°C, and the supernatant collected. Bradford Protein assay method was used to determine the protein concentration using bovine serum albumin (BSA) as the standard (Bio-Rad catalog 500-0006). 1-20µg of tissue protein to be analyzed were combined with equal parts 2X Laemmli sample buffer (Bio-Rad, catalog 161-0737) containing 10% β-mercaptoethanol, and then boiled for 15 minutes, loaded onto a reducing-denaturing SDS-polyacrylamide gel (10-12% resolving, 4% stacking), and one-dimensional electrophoresis was carried out for 4 hours at 75-100 volts in a Tris-glycine-SDS (Bio-Rad, catalog 161-0732) running buffer. Proteins were transferred to a 0.45µm nitrocellulose membrane (Bio-Rad, catalog 162-0115) in Tris-glycine transfer buffer containing 20% methanol cooled to 4°C. After transfer, the membrane was blocked overnight at 4°C using 5% non-fat dry milk (Bio-Rad, catalog 170-6404) in Tris-buffered saline pH 7.3, 0.1% Tween 20 (TBSTw) with agitation. Blots were incubated for 1 hour at room temperature with a 1:2000 dilution of a rabbit anti-*Pax6* primary antibody directed against the C-terminus of the protein (Covance, catalog PRB-278P) in blocking solution with gentle agitation. Following primary antibody incubation, membranes were rinsed several times in 1xTBSTw and incubated with goat anti-rabbit HRP-conjugated secondary antibody (1:20,000; Bio-Rad, catalog 179-5046; or 1:10,000; Santa Cruz, catalog sc-

2004) in 5% non-fat dried milk for 1 hour at room temperature with gentle agitation. After removal of the secondary antibody, the membranes were rinsed several times in TBSTw. For signal detection, Immuno-Star Western C Kit (BioRad catalog 170-5070) was used, followed by manual autoradiograph development. To determine protein loading, membranes were subsequently stripped (32), blocked in 10% milk in 1xTBSTw overnight at 4ºC and incubated with rabbit anti-GAPDH primary antibody (1:1000, abcam, catalog ab9495) and processed as described above.

Flow Cytometry

Freshly dissected pituitary glands from adult mice (6-8 months of age) were then extracted and mechanically dissociated in 1x PBS followed by fixation in 4% PFA. Fixed cells were then permeabilized in 90% methanol for 30 minutes on ice, followed by several washes in 1xPBS, leaving 100ul of solution in each wash. Cells were then stained according to established protocols for immunofluorescence staining of cells for flow cytometry (33,34) using the same primary antibodies listed above for immunofluorescence and goat-anti rabbit allophocyan (APC) for secondary antibody. APC fluorescence was detected through 665/20 filter with logarithmic amplification. Cells were initially gated on a scatter plot and a FSC pulse width vs. FSC peak plot to eliminate debris and doublets, respectively. 30,000 events were collected for each sample. Acquisition was performed using a CyAn ADP Analyzer (Beckman Coulter, Hialeah, Florida) and data analyzed using FlowJo software version 9.3.1 (Treestar, Inc., Ashland, Oregon).

MRI Data Collection and Analysis

All MRI protocols and animal procedures were approved by the IACUC at the University of Georgia prior to data collection. Adult mice heterozygous for the *Small eye Neuremerg* allele

(4-5 months of age, 4 male, N=9) and wild-type littermates (4-5 months of age, 4 male, N=9) were imaged on a 7T Agilent MRI system using a 10mm surface coil. T2-weighted 3D structural images were acquired for each animal (fast spin echo T2 weighted MRI: TR/TE 700/30 msec, with FOV of 20 x 20 x 20 mm, 256^3 matrix, 4 averages, scan time 45 minutes). Voxel-based morphometry (VBM) (35) analysis was performed using Statistical Parametric Mapping Software (SPM8; Wellcome Trust Centre for Neuroimaging, http://www.fil.ion.ucl.ac.uk/spm/) run on a MATLAB software platform (MATLAB Release 2015b, The Mathworks, Inc., Natick, Massachusetts, United States). All 3D images were skull stripped, aligned, and co-registered with a mouse brain template. Segmentations were performed on processed images to separate white matter, grey matter and cerebro-spinal fluid (CSF) images. The pituitary gland notably segmented with CSF, and these images were smoothed with a 0.2-mm FWHM (Full Width Half Maximum) Gaussian kernel and compared with two-sample *F*-tests. ($p<0.05$ cluster size of 100 were used and widely accepted as statistically significant). Manual tracings of the pituitary were performed using the Aedes software package (http://aedes.uef.fi) (36) and whole pituitary size as measured in number of pixels was compared using a 2-sample t-test.

Weight data

To obtain weight data, *Pax6*$^{+/-}$ mice and their wild type littermates were euthanized and weighed immediately after euthanasia. The mice were 6 to 9 months of age. For each genotype 76 male mice and 79 female mice were weighed. For both males and females, equal numbers of wild-type and *Pax6*$^{+/-}$ mice were weighed at each age (males/females, 6 months: 35/33; 7 months: 14/8; 8 months: 13/6; 9 months: 13/32). Data was analyzed by Student's t-test.

Radioimmunoassays

Blood serum was isolated from individual adult *Pax6*$^{+/-}$ mice and wild-type littermates (6 males and 4 females per genotype, 4 months old) using BD serum isolation tubes. Samples were then frozen and sent to Michigan State University endocrine diagnostic labs to measure concentration of total T_3 and T_4. Data was analyzed by Student's t-test.

Results

Pax6 is expressed in the adult anterior pituitary gland

Although previous studies have reported on *Pax6* expression in the developing pituitary gland (3,20), there have been no reports of *Pax6* expression in the adult pituitary gland. Therefore, we examined adult pituitary glands from wild-type mice to determine if *Pax6* expression continues throughout adulthood. The mouse *Pax6* gene generates several different mRNAs and encodes for three protein isoforms (25). Using a probe designed to detect all *Pax6* transcripts, we found by RNA *in situ* hybridization that *Pax6* is expressed in the adult anterior pituitary but not in the intermediate or posterior lobes (Figure 2.1A) as was previously reported in the E18.5 embryo (3). Comparison of the RNA *in situ* hybridization signal observed in pituitary to eye tissue suggested that there was a much lower level of *Pax6* expression in the pituitary compared to cells in the retina (data not shown). To test this, reverse transcription polymerase chain reactions (RT-PCR) were performed on total RNA prepared from whole eye and pituitary under semi-quantitative conditions (Figure 2.1B, data not shown). *Pax6* transcripts were more abundant in total RNA prepared from whole eye than from pituitary. Although the RT-PCR experiments only capture bulk expression and will be strongly influenced by the numbers of cells that express the gene compared to those that do not within the different tissues, this data when combined with the RNA *in situ* data suggests that *Pax6* is expressed more or less uniformly by cells in the anterior pituitary and that the level of expression per cell is lower than those of the retina. Western analysis revealed the presence of the 46 kDa canonical PAX6 protein and the 48 kDa alternatively spliced exon 5a isoform (PAX6+5a) in the pituitary, albeit at lower levels than in the eye (Figure 2.1C). Together these results demonstrate that *Pax6* is expressed in the anterior pituitary gland in adult mice and suggest that, in addition to its role in the

development of the pituitary gland, *Pax6* also likely plays a maintenance role in the adult anterior lobe.

Pax6$^{Seu\text{-}Neu}$ is required for normal morphological pituitary development

Pax6 is known to be required for pituitary development. In *Pax6* homozygous null mice, there is a significant decrease in somatotropes and lactotropes in the anterior pituitary (20,21); however, potential modifier effects have been observed (21) and the net effect of *Pax6* dosage on pituitary development in neonates has not been reported. To provide a starting point for assessing potential changes in the adult pituitary of the *Pax6*$^{+/-}$ allele used the current study, we first examined by histology the pituitaries of neonate littermates at birth (Figure 2.2). Because mice heterozygous null for *Pax6* die at birth, this was the oldest developmental time point at which we could compare pituitary morphology between *Pax6*$^{+/+}$, *Pax6*$^{+/-}$ and *Pax6*$^{-/-}$ animals. Using the trigeminal ganglion as an anatomical landmark, we examined histological sections cut through the middle of the pituitary for each genotype. The H&E stained pituitary tissue sections from *Pax6*$^{+/-}$ neonates appear comparable to those from *Pax6*$^{+/+}$ neonates (compare Figure 2.2B to A). For both genotypes, the three lobes of the pituitary are clearly identifiable. The posterior lobe is located directly below the third ventricle of the brain and is adjacent to a clearly delineated intermediate lobe and the lumen separating it from the anterior lobe (Figure 2.2A,B). The lobes appear comparable in size (Figure 2.2A,B; data not shown). In contrast, sections cut through the *Pax6*$^{-/-}$ gland showed significant differences in the presumptive posterior pituitary lobe and the presumptive intermediate lobe was not distinct (compare Figure 2.2C to A). Notably in the null mutants, the cells in the presumptive posterior lobe of the pituitary exhibit histological characteristics similar to the anterior lobe, but do not express anterior lobe cell type markers (see Figure 2.4). The anterior lobe of the pituitary in *Pax6*$^{-/-}$ neonates was histologically more

comparable to the anterior lobe present in the other genotypes, but appeared to be thicker in the area directly below the posterior lobe than was observed in either wild-type and *Pax6*$^{+/-}$ littermates (compare Figure 2.2C to A,B). These results demonstrate that the histological development of the pituitary is comparable between *Pax6*$^{+/+}$ and *Pax6*$^{+/-}$ mice at P0 and establish a baseline for the adult and cellular studies.

Pax6$^{+/-}$ pituitaries are morphologically normal but enlarged in adult mice

Since *Pax6* is semidominant and, in some organs such as the eye, the reduction in functional protein results in a change in morphology, we thought it was plausible that the structure and morphology of the mutant pituitary glands might also be affected. At both the gross and histological level, the pituitaries in adult *Pax6*$^{+/-}$ mice appeared comparable to those in wild-type littermates (Figure 2.3A-D). The anterior, intermediate and posterior lobes could be clearly identified both *in situ* and in histological sections.

Casual inspection of the pituitaries *in situ*, in dissected whole tissues, and in sectioned tissues however suggested that the anterior lobe of the pituitaries from the *Pax6*$^{+/-}$ mice were slightly, but noticeably, larger than those from wild-type littermates. To avoid possible confounds associated with measuring freshly dissected tissues or changes in size associated with fixation and histology, we utilized MRI as a means of determining pituitary volume in live adult *Pax6*$^{+/-}$ mice and wild-type littermates. An increase of pituitary size was detected in adult *Pax6*$^{+/-}$ mice compared to wild-type littermates by high-resolution 3D MRI imaging (Figure 2.3E,F). Voxel-wise analysis suggested this increase in a large portion of tissue in the pituitary (Figure 2.3E), and the significant increase in size was confirmed by manual tracing and ROI analysis (Figure 2.3F, p=.00067).

Collectively, these data show that although there is no gross difference in morphology of the pituitary glands of heterozygous mutant mice at birth, there is appears to be a significant increase in the size of the anterior lobe during adulthood as evidenced by MRI analysis.

Expression of endocrine cells in neonatal mice

In mice, pituitary hormone cell types develop in a dorsal to ventral pattern, with corticotropes (ACTH) dorsal-most, followed by somatotropes (GH) and lactotropes (PRL) in the intermediate position, thyrotropes (TSH) in the ventral-intermediate position and gonadotropes (FSH and LH) ventral-most. By E17.5, all cell types are present and have begun to take on their adult locations. Previous studies reported a role for *Pax6* in the dorsal to ventral patterning of the anterior pituitary gland, where the complete absence of *Pax6* resulted in a dorsal expansion of ventral cell types (gonadotropes and thyrotropes) at the expense of the more dorsal somatotropes and lactotropes (20,21). To determine if these same changes occur in heterozygous mutants, we analyzed the pituitary glands of newborn mice of all three genotypes, comparing heterozygous mutants to both wild-type and homozygous mutants. Due to nature of the symptoms reported by people with aniridia, it seemed unlikely that PRL would be causal for these symptoms and our results for FSH were not interpretable due to the fact that it is a cyclical hormone. Therefore, we focused our analysis on 4 of the 6 anterior pituitary hormone cell types: ACTH, GH, TSH, and LH.

To quantify the relative numbers of each of these hormone-producing cell types in the anterior pituitaries of $Pax6^{+/+}$, $Pax6^{+/-}$ and $Pax6^{-/-}$ neonates were serially sectioned in their entirety and separately immunolabeled for ACTH, GH, TSH or LH (Figure 2.4). The total numbers of specifically immunolabeled cells for each hormone were systematically counted in every other section so that for any given experiment about half the total number of cells in each

anterior pituitary were assessed with respect to hormone cell type. The average number and standard deviation (SD) of immunolabeled cells per genotype were determined using three or four animals per group (Figure 2.4 D, H, L, P). Statistical comparison between genotypes was performed by analysis of variance (ANOVA). There were no significant changes in ACTH-cell numbers in either the heterozygote mutant or the homozygote mutant compared to wild-type littermates (Figure 2.4A-D). For neonates at P0, wild-type pituitary glands had an average of 3,622±444 ACTH expressing cells while *Pax6*$^{+/-}$ and *Pax6*$^{-/-}$ glands had averages of 4,217±380 and 3,964±962 expressing cells, respectively (Figure 2.4D). Significant changes in the numbers of GH- and TSH-expressing cells in the neonatal pituitaries did exist between the different genotypes (Figure 2.4H,L). The numbers of GH-expressing cells was decreased in both homozygous (5,574±1442 cells) and heterozygous (6,959±1103 cells) mutant genotypes compared to wild-type littermates (9,225±1049 cells). Conversely, the numbers of TSH-expressing cells was increased in both homozygous (5,854±810 cells) and heterozygous (4,828±817 cells) mutant genotypes compared to wild-type littermates (2,803±917 cells). The numbers of LH-expressing cells were generally numerically increased in both *Pax6*$^{-/-}$ (2,965±731 cells) and *Pax6*$^{+/-}$ (2,400±1448 cells) animals compared to wild-type (1883±372 cells), but the differences were not statistically significant ($p = 0.44$), largely due to the variance observed in the heterozygous samples (Figure 2.4P).

The comparative results for ACTH-, GH- and TSH-expressing cells between wild-type and homozygous mutant neonates are consistent with those reported for the embryonic pituitary (20,21). Interestingly, there are also differences in both GH- and TSH-expressing cell numbers in heterozygotes compared to wild-type, and the trends observed across genotypes suggests that the effect is *Pax6* dosage sensitive. Our results for LH-expressing cells appears to be inconsistent

with the report by Kioussi et al. that there was a significant increase in the numbers of LH-expressing cells in homozygous mutant embryos relative to wild-type, but it is consistent with the LH data shown in the study by Bentley et al (21). The apparent discrepancies for this cell type may be due differences in the markers used (LH here and by Bentley et al; SF-1 by Kioussi et al), problems of sample size, or potential modifier effects between the mice used in these studies.

Endocrine cell numbers in adult pituitary glands

To better understand the effect of *Pax6* haplo-insufficiency on pituitary function in adult mammals, flow cytometry was used to quantify the relative numbers of ACTH, GH, TSH or LH hormone-producing cell types in the anterior pituitaries of adult $Pax6^{+/+}$ and $Pax6^{+/-}$ mice (6-8 months of age). There was no significant difference in the numbers of ACTH-, GH-, or LH-expressing cells between wild-type and heterozygous mutant mice (Figure 2.5). On average, 13% of cells positively stained for ACTH in wild-type pituitary glands and 11% in $Pax6^{+/-}$ pituitary glands; 34% of cells positively stained for GH in wild-type glands and 31% in those of $Pax6^{+/-}$ mice; and 9.6% positively stained for LH in wild-type mice compared to 7% in mutants. In contrast, the numbers of TSH-producing cells were significantly increased in $Pax6^{+/-}$ pituitaries compared to wild-type (Figure 2.5 K-O). On average, 19% of cells positively labeled for TSH in $Pax6^{+/-}$ mice compared to 11% in wild-type.

Assessing the effects of increased number of TSH-producing cells in $Pax6^{+/-}$ mice

A possible effect of the increase in TSH-producing cells in the pituitaries of *Pax6* heterozygous mutant mice is a change in metabolism. This intrigued us because many patients with aniridia self-report difficulty managing their weight (16). Therefore, we first decided to measure the weights of $Pax6^{+/-}$ mice compared to their wild-type littermates. The mice were

between 6 to 9 months of age and housed under directly comparable conditions. We found that as a group *Pax6*$^{+/-}$ mice weighed less than wild-type for both males and females. Weights for wild-type males averaged 46.0 ± 6.8g compared to average weight of 43.6 ± 6.8g for *Pax6*$^{+/-}$ mice (Figure 2.6A, n= 76 *Pax6*$^{+/+}$ and 76 for *Pax6*$^{+/-}$, p = 0.03). Similarly, wild-type females weighed an average of 36.5 ± 7.0g and *Pax6*$^{+/-}$ females weighed an average 33.7 ± 7.0 (Figure 2.6B, n= 79 *Pax6*$^{+/+}$ and 79 for *Pax6*$^{+/-}$, p = 0.01). Interestingly, there was a larger variation in the weights observed for both male and female *Pax6*$^{+/-}$ mice compared to their wild-type littermates (Figure 2.6A,B). These data suggest that metabolic control mechanisms and/or tolerances may be altered in these *Pax6*$^{+/-}$ mice compared to wild-type animals.

Given our finding that TSH cell numbers were increased in *Pax6*$^{+/-}$ mice and that thyroid hormones are known to be involved in the regulation of basal metabolism and thermogenesis (37–40), we wanted to assess the levels of TSH and thyroid hormones in these mice. TSH acts on the thyroid gland to stimulate release of triiodothyronine (T_3) and thyroxine (T_4). Because access to a validated TSH assay for mice proved problematic, we report only on our findings for circulating levels of T_3 and T_4, which also can provide a readout of TSH levels. The average concentration of T_3 in *Pax6*$^{+/-}$ mice was 0.7 ± 0.2 nmol/L, slightly higher than the average concentration for wild-type of 0.6 ± 0.1 nmol/L, although this difference was not statistically significant (Figure 2.6C, p = 0.26). For T_4, the average concentration for *Pax6*$^{+/-}$ mice was 65 ± 6 nmol/L, significantly higher than the average concentration for wild-type of 59 ± 6 nmol/L (Figure 2.6D, p = 0.04). Although there is no notable difference in levels of T_3, the elevated concentration of T_4 in *Pax6*$^{+/-}$ mice may lead to an increased metabolism, which may explain why our heterozygous mutant mice weigh significantly less than their wild-type littermates.

Discussion

Recent studies have suggested that adult patients with aniridia have a greater propensity for unexplained systemic symptoms, such as infertility, severe eczema and issues with weight, which may be related to *PAX6* haploinsufficiency (16). The goal of this study was to test if any changes were present in the adult anterior pituitary gland as a result of a heterozygous mutation in the *Pax6* gene in mice and, if so, whether or not these changes could explain some of the symptoms experienced by patients with aniridia.

This study represents the first report of *Pax6* expression and function in the adult pituitary gland. We found that *Pax6* expression is maintained in the anterior gland in adult mice, and expression is no longer restricted to the dorsal region, but is expressed throughout the anterior lobe much like it is at E14.5. This suggests that the maintenance of *Pax6* expression may be required for proper functioning of the anterior pituitary as it is in the pancreas. In the developing pancreas, cells of the endocrine lineage express *Pax6,* and a complete loss of *Pax6* results in both lack of development of α-cells and a marked decrease of all other cells types in the endocrine pancreas (41). In the adult, *Pax6* is expressed throughout the islets of Langerhans and continued expression is required for normal pancreatic function (4). In the case of the pituitary, *Pax6* is not required for specification of the hormone producing cells, as evidenced by the fact that in the complete absence of *Pax6* all cell types still develop, but *Pax6* is required for generation of normal numbers of somatotropes, lactotropes and thyrotropes (current study (20,21)), and could play a role in the ability of the adult pituitary to adjust to physiological demand. This could occur at the level of endocrine cell function or in mediating changes in cell numbers. Changes in physiological demand have been shown to result in changes the populations of hormone-producing cells in the adult pituitary, and this can occur through proliferation of

terminally differentiated cells; transdifferentiation of differentiated cells, such as conversion of somatotrophs to lactotrophs; and/or differentiation of progenitors/stem cells (42). In the nervous system, *Pax6* is known to regulate potency (43), cell-cycle and differentiation kinetics (44–50), the balance between neural stem cell self-renewal and neurogenesis (51), and is required to properly respond to signals during lens induction (52). In the context of endocrine cell function, *Pax6* is implicated in the normal function of intestinal L cells by direct activation of the *glucagon-like peptide 1* gene (5–7), and of islet cells by regulation of a number of genes required for endocrine cell function, including the production of insulin, glucagon and somatostatin (23,53–57). Thus, it is plausible that a reduction in *Pax6* protein levels could affect pituitary function in adult mammals.

Significantly, we found an increase in the numbers of TSH-producing cells in both neonatal and adult mice heterozygous null for *Pax6*, and this correlated with a significant increase in circulating levels of T_4 in adult animals. An increase of T_4 levels in the serum above normal was consistent with a putative increase in the amount of TSH being produced by the pituitaries in *Pax6*$^{+/-}$ animals. When stimulated by TSH, the thyroid gland releases T_4 and T_3 into the blood (58–65). An increase in circulating levels of T_4 would be expected to also lead to a concomitant increase in the levels of T_3 in the brain, liver, kidney, intestines and other target tissues (63,66–75). In rodents and other mammals, T_3 is estimated to be 3 to 5 times more potent than T_4 (76–79). Thus, a modest increase in circulating T_4 levels could result in a significant change in the metabolic potency of T_4 in target tissues due to its conversion to T_3.

While the physiological effects of abnormally high levels of circulating T_4 in *Pax6*$^{+/-}$ mice were not fully assessed, one possibility was that these animals were exhibiting symptoms associated with mild hyperthyroidism. Such changes could include increased appetite, increased

metabolism, tachycardia, heat intolerance, fatigue, and anxiety and irritability. Consistent with this hypothesis, we found that both male and female *Pax6*$^{+/-}$ adult mice weighed less than their wild-type littermates housed under identical conditions. Similar results were obtained in two other studies examining the role of *Pax6* in metabolic homeostasis (7,80), and the authors noted that although adult *Pax6*$^{+/-}$ mice showed increased food intake compared with wild-type control mice, they were resistant to diet-induced fat accumulation (7).

It may be difficult, however, to disentangle changes specifically associated with elevated levels of T_4 from *Pax6* mediated changes in other metabolic systems. In addition to its role in the development and function of the eye and brain, *Pax6* is known to be required for normal development and function of the endocrine pancreas (4,41), and it has been implicated in the normal development and/ or function of the enteroendocrine cells that line the intestine (5–7). Given that metabolic pathways are tightly regulated, and that changes in *Pax6* can affect the development and/or function of the pituitary (current study, (20,21)), pineal gland (81,82), pancreas (4,41,56,83–86), enteroendocrine L-cells (5–7), and neuroendocrine neurons in the hypothalamus (23), it is likely that the physiological effects of increased thyroid hormone activity will act synergistically and/or additively to *Pax6*-dependent changes in these other metabolic pathways. Two tissues that bear closer examination are the liver and the kidney, where chronically elevated levels of thyroid hormones can result in clinically important alterations the function of these organs (87,88). In addition to possible effects in the adult, elevated levels of T_4 could impact cortical development directly (89,90).

Although we found that as a group *Pax6*$^{+/-}$ adult mice weighed less than *Pax6* normal mice, individuals with aniridia report a higher incidence of obesity as adults (16). While this apparent discrepancy could be due to inherent metabolic differences between rodents and

humans, it could also reflect changes in the metabolic control mechanisms and/or tolerances associated with a reduction in functional *Pax6* protein. Consistent with this idea, we noted a larger variation in the weights observed for both male and female *Pax6*$^{+/-}$ mice compared to their wild-type littermates, and some of the female *Pax6*$^{+/-}$ mice were much heavier than wild-type littermates (Figure 2.6B). Thus, while *Pax6*$^{+/-}$ mice maintained on normal rodent chow exhibited lower body weights as a group, these animals may exhibit a higher BMI compared to wild-type animals when provided access to a calorically rich diet. In a recent report, *Pax6*$^{+/-}$ mice fed a high fat diet gained more weight than wild-type control mice (Figure 1A in (80)); these mice also exhibited insulin resistance, reduced prohormone convertase 1/3 production, and increased proinsulin secretion (80). A possible link between *Pax6* function and obesity in the general population has been reported (91). It is important to note that not all *Pax6*$^{+/-}$ mice or persons with aniridia exhibit a high BMI, and that in general both juvenile mice and children with aniridia do not appear to be overweight (Johnson and Lauderdale, unpublished).

In addition to an increase in TSH-labeled cells, *Pax6*$^{+/-}$ neonates exhibited a reduction in the number of GH-labeled cells in the pituitary. We initially thought that the increase in TSH-producing cells was at the expense of GH-producing cells, consistent with the model proposed by Kioussi *et al*. (20). However, this seems unlikely since the number of GH-producing cells increased to normal in adult *Pax6*$^{+/-}$ mice, suggesting that GH cell fate determination was delayed or subject to a compensatory mechanism. Consistent with this idea, Bentley, *et al*. (21) found that at E17.5 GH serum levels were five-fold higher in wild-type embryos compared to *Pax6* null embryos, but that this difference was reduced to three-fold by P0. During normal pituitary organogenesis, there is an increase after birth in the numbers of the different hormone producing cell types and this expansion is driven by the hypothalamic releasing hormones and by

physiological demands (42). In the case of somatotropes, this increase involves reentry into the cell cycle (92), and interestingly, requires thyroid hormone. Although young adult mice deficient in thyroid hormone production exhibit a 4-fold decrease in the numbers of GH-expressing cells in their pituitaries, treatment of these animals with T_4 from birth increases GH cell numbers to those expected in normal age-matched animals (93). While the mechanism underlying the recovery of normal numbers of GH-expressing cells in the adult pituitary of *Pax6*$^{+/-}$ mice is not known, it may be driven in part by elevated levels of T_4. Regardless of the mechanism, the recovery of GH cells would explain why *Pax6*$^{+/-}$ mice do not show any of the physical attributes associated with a decrease in GH.

Three recent studies have examined pituitary function in aniridia patients with heterozygous *PAX6* mutations (22–24). The first study performed an endocrinological evaluation on related individuals with aniridia, which consisted of a family with two affected individuals and a second family with 36 members, distributed in five generations, of whom 14 members were affected (22). These individuals were tested for FSH, LH, ACTH, prolactin, GH, somatomedin C, TSH, cortisol, estradiol concentrations, synacthene, and luteinizing hormone-releasing hormone (LHRH). Of these individuals, endocrine changes were detected in one person with aniridia from the first family (low ACTH concentration) and in two individuals with aniridia in the second family (reduced response to synacthen/ACTH test). The second study examined a pedigree with 19 individuals with aniridia spread over three generations and reported that the levels of ACTH (the only pituitary hormone assessed) and α-MSH were decreased in the blood of the aniridia patients as a group (23). The third study reported a single case, which was that of a 40 year old women who had obesity, exhibited an impaired glucose tolerance with a delayed insulin response, and also exhibited slightly impaired pituitary function, which consisted

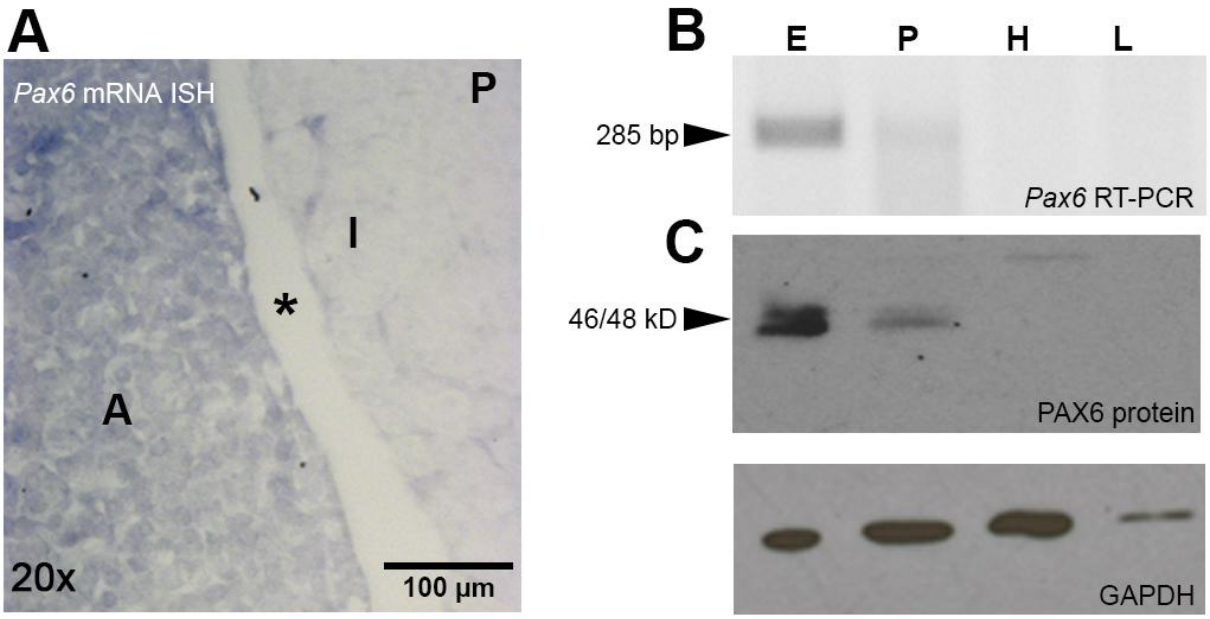
of subclinical hypogonadotropic hypogonadism and borderline GH deficiency, but apparently normal ACTH levels (24).

One conclusion that can be drawn from these studies is that individuals with *PAX6*-mediated aniridia do not necessarily exhibit overt clinical symptoms associated with abnormal pituitary function. The reduction in ACTH levels can be attributed to a PAX6-dependent reduction in the levels of prohormone convertase (PC1/3) expressed in the hypothalamus (23). PC1/3 is the enzyme responsive for converting pro-opiomelanocortin (POMC) into ACTH, which is further converted into α-MSH (94). In the case of the single individual with aniridia exhibiting somewhat reduced levels in FSH, LH, and GH, but apparently normal levels of ACTH, the changes could be explained by a mechanism unrelated to *PAX6* or may reflect phenotypic variation.

When compared to our findings in mice, it is possible that *Pax6* haploinsufficiency affects the human pituitary differently than in rodents; however, we think it likely that *Pax6* haploinsufficiency affects mammalian pituitaries in similar ways. Our studies show that, in mice, a loss of function mutation in *Pax6* results in increased numbers of TSH-producing cells in the developing and adult pituitary gland, leading to an increase in circulating concentration of total T_4 as measured in a fairly homogeneous group of mice. It is possible that there are comparable changes in persons with aniridia, but that there is more phenotypic variability. As described above, background-dependent variation particularly in somatotropes and lactotropes, but also corticotropes and thyrotropes, have been observed in mice (21). In the case of ACTH, although we did not find a change in corticotrope cell numbers between $Pax6^{+/-}$ and wild-type animals, $Pax6^{+/-}$ mice are reported to have a reduction of ACTH and α-MSH in hypothalamic tissue similar to humans with aniridia (23).

In conclusion, several lines of evidence indicate that the *Pax6* gene plays a role in the development and function of the endocrine system, and that the normal function of this system can be perturbed by *Pax6*-haploinsufficiency. Continued investigation into this important question is needed if we are to understand the systemic aspects of aniridia.

Acknowledgments
The authors thank Drs. Sally Camper and Peter Gergics (Department of Human Genetics, University of Michigan Health System) and Dr. Shannon Davis (Department of Biological Sciences, University of South Carolina) for helpful discussions and technical advice on tissue preparation of neonate pituitary glands and immunolabeling cells in the pituitary, and Dr. Julie Gordon (Department of Genetics, University of Georgia) for helpful discussion and comments on the manuscript. Confocal microscopy was performed using the services of the Biomedical Microscopy Core under the direction of Dr. Muthugapatti K. Kandasamy. Flow cytometry was performed using the facilities of the CTEGD Cytometry Shared Resource Lab under the direction of Ms. Julie Nelson. Magnetic resonance imaging was performed using the Agilent Magnex 7 Tesla small animal MRI system in the UGA Bio-imaging Research Center (BIRC). The authors thank A. F. Parlow at the National Hormone and Peptide Program (NHPP), Harbor-UCLA Medical Center (Torrance, CA 90509, USA), for providing antibodies used in this study, and the Michigan State University Diagnostic Center for Population and Animal Health (Lansing, MI 48909-7576, USA) for assessing serum levels of T_3 and T_4.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Pax6 mRNA ISH
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20x
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C
46/48 kD
PAX6 protein
GAPDH

Figure 2.1

Figure 2.1: *Pax6* expression persists at low levels in adult pituitary glands.
(A) Low levels of *Pax6* mRNA present in anterior pituitary gland as shown by RNA in situ hybridization. Abbreviations: Anterior pituitary (A), intermediate pituitary (I), posterior pituitary (P); scale bar = 100µm. (B) *Pax6* transcripts containing the alternatively spliced exon 5a were detected in total RNA prepared from the eyes (E) and pituitaries (P) of adult mice, but not heart (H) or lung (L). (C) Western blot shows presence of full-length PAX6 protein with (48 kDa) and without exon 5a (46 kDa) in lysates prepared from eye and pituitary, but not heart or lung. *In situ* hybridization and Western analyses were performed on tissues collected from 7 months old mice. RNA for the RT-PCR experiments was collected from 9 months old mice.

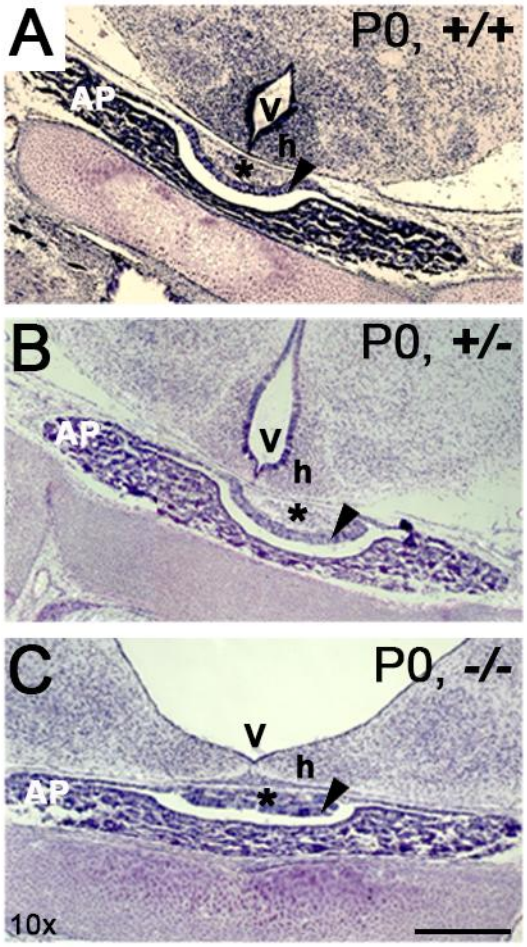


Figure 2.2

Figure 2.2: *Pax6*$^{Sey\text{-}Neu}$ is required for normal pituitary development.
Histological comparison of the pituitaries in *Pax6*$^{+/+}$ (A), *Pax6*$^{+/-}$ (B), and *Pax6*$^{-\backslash-}$ (C) littermates at P0 age. The H&E stained pituitary tissue sections from *Pax6*$^{+/-}$ neonates appear comparable to those from *Pax6*$^{+/+}$ neonates, while those from *Pax6*$^{-/-}$ neonates show differences in the presumptive posterior (asterisk) and intermediate (arrowhead) pituitary. The enlarged ventricle in *Pax6*$^{-/-}$ neonates is due to aberrant brain development. AP, anterior pituitary; v, third ventricle; h, hypothalamus; asterisk denotes position of posterior pituitary, arrowhead denotes intermediate pituitary. Scale bar = 100μm. N=3 for each genotype

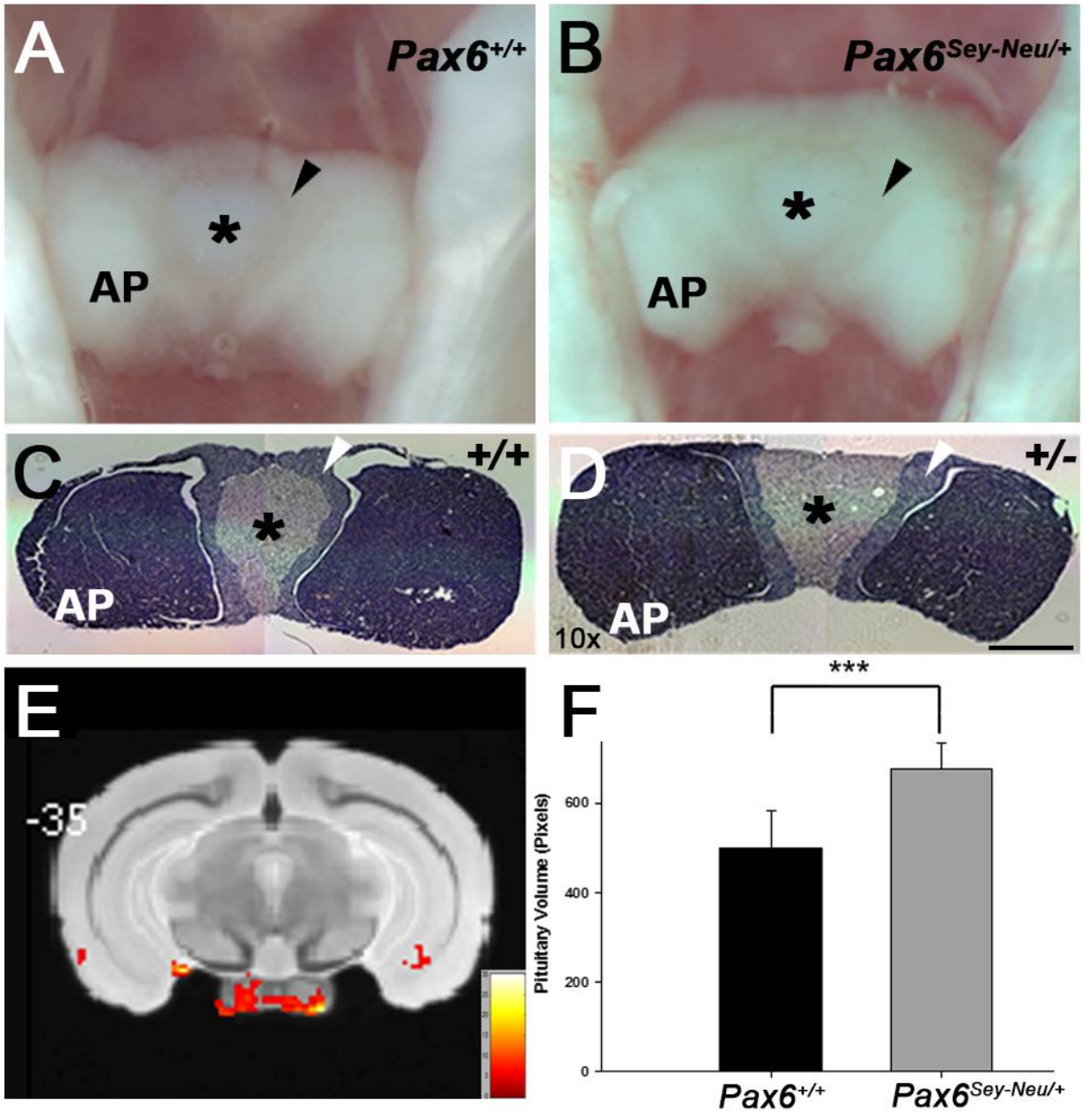
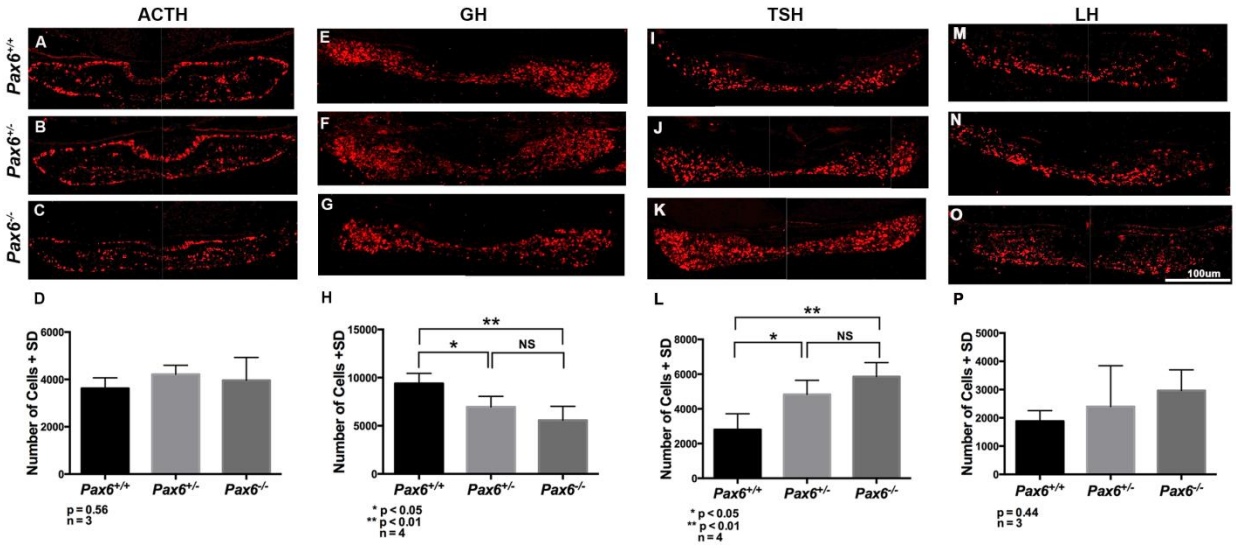

A
Pax6+/+
AP
B
Pax6Sey-Neu/+
AP
C
+/+
AP
D
+/-
AP
10x
E
-35
F
***
Pituitary Volume (Pixels)
600
400
200
0
Pax6+/+
Pax6Sey-Neu/+

Figure 2.3

Figure 2.3: *Pax6*$^{+/-}$ pituitary glands show no gross morphological differences compared to those of *Pax6*$^{+/+}$ mice but are enlarged.
Comparison of pituitaries in *Pax6*$^{+/+}$ (A,C) and *Pax6*$^{+/-}$ (B,D) littermates at 8 months of age. (A,B) pituitaries *in situ*. (C,D) H&E stained transverse sections cut through the middle of the pituitary. The pituitary glands were comparable between genotypes. N=6 animals, 3 male and 3 female, for each genotype; animals were 6-8 months of age. AP, anterior pituitary; asterisk denotes posterior pituitary; arrowhead denotes intermediate pituitary. Scale bar = 100um. (E,F) Voxel-Based Morphometry (VBM) results of CSF in a 1.5mm section of adult *Pax6*$^{+/-}$ compared to wild-type littermate mouse brains at 4-5 months of age (*Pax6*$^{+/+}$, n=9, *Pax6*$^{+/-}$, n=9). Section number -35 relative to position of Bregma. Red pixels denote regions of statistical difference ($p<.001$) between wild-type and *Pax6*$^{+/-}$ mice; differences are mapped to wild-type brain; psuedo color map – t-scale values 0 to 30. (F) Pituitary size in pixels in wildtype and *Pax6*$^{+/-}$ mice via ROI analysis from 3D T2 MRI (*Pax6*$^{+/+}$, n=9, *Pax6*$^{+/-}$, n=9; 2 group t-test; ***=$p<.001$).


ACTH
GH
TSH
LH
Pax6+/+
Pax6+/-
Pax6-/-
A
B
C
E
F
G
I
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N
O
100um
D
H
L
P
Number of Cells + SD
Number of Cells +SD
p = 0.56
n = 3
* p < 0.05
** p < 0.01
n = 4
p = 0.44
n = 3
*
**
NS

Figure 2.4

Figure 2.4: Reduction in *Pax6* results in an increase in TSH-producing cells in neonate mouse pituitary glands.

Representative pituitary tissue sections from wild-type (A,E,I,M), *Pax6*$^{+/-}$ (B,F,J,N) and *Pax6*$^{-/-}$ (C,G,K,O) P0 neonates showing expression of ACTH (A-C), GH (E-G), TSH (I-K), and LH (M-O). Quantification and comparison of the relative numbers of ACTH (D), GH (H), TSH (L) and LH (P) expressing cells in the pituitary of each genotype reveals that there is both an increase in TSH producing cells and a decrease in GH producing cells in both *Pax6*$^{-/-}$ and *Pax6*$^{+/-}$ mutant mice relative to wild-type. *$p<0.05$, **$p<0.01$. Data represented as mean ± std dev, n=3 for ACTH and LH, n=4 for TSH and GH for each genotype.

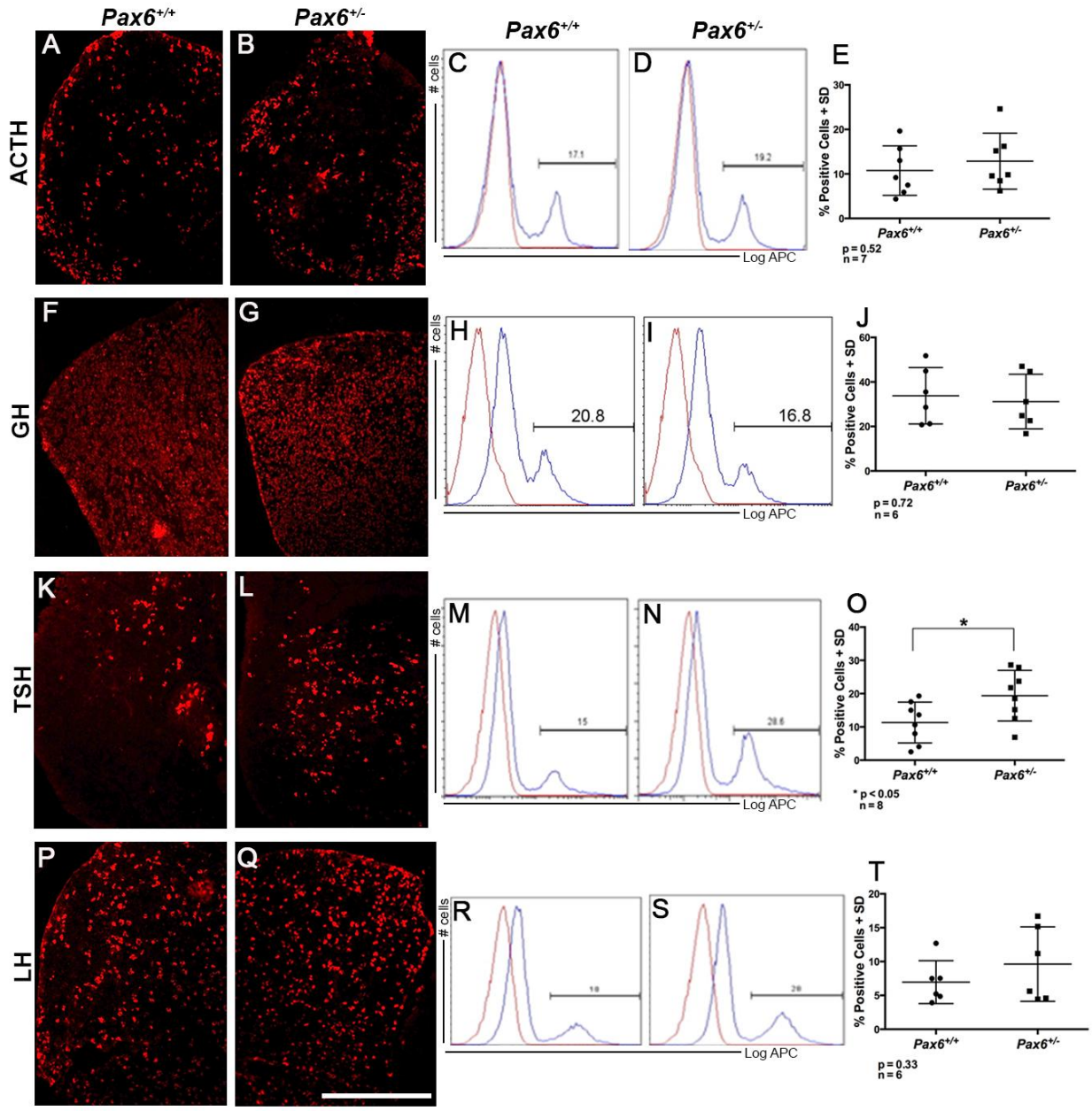
Pax6+/+
Pax6+/-
ACTH
GH
TSH
LH
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
cells
Log APC
17.1
19.2
20.8
16.8
15
28.6
19
28
% Positive Cells + SD
p = 0.52
n = 7
p = 0.72
n = 6
* p < 0.05
n = 8
p = 0.33
n = 6

Figure 2.5

Figure 2.5: Increase in TSH-producing cells in *Pax6*$^{+/-}$ mice persists in adult pituitary glands.

Representative pituitary tissue sections from wild-type (A,F,K,P) and *Pax6*$^{+/-}$ (B,G,L,Q) adult mice showing expression of ACTH (A,B), GH (F,G), TSH (K,L), and LH (P,Q). Representative results from flow cytometry performed on pituitaries from wild-type (C,H,M,R) and *Pax6*$^{+/-}$ (D,I,N,S) adults sorted for ACTH (C,D), GH (H,I), TSH (M,N), and LH (R,S). Quantification and comparison of the relative numbers of ACTH (E), GH (J), TSH (O) and LH (T) expressing cells reveals that there is an increase in the numbers of TSH-expressing cells in the pituitaries of *Pax6*$^{+/-}$ animals compared to wild-type. Data represented as mean ± std dev. Scale bar = 100um. Mice were 6-8 months old and include both sexes.

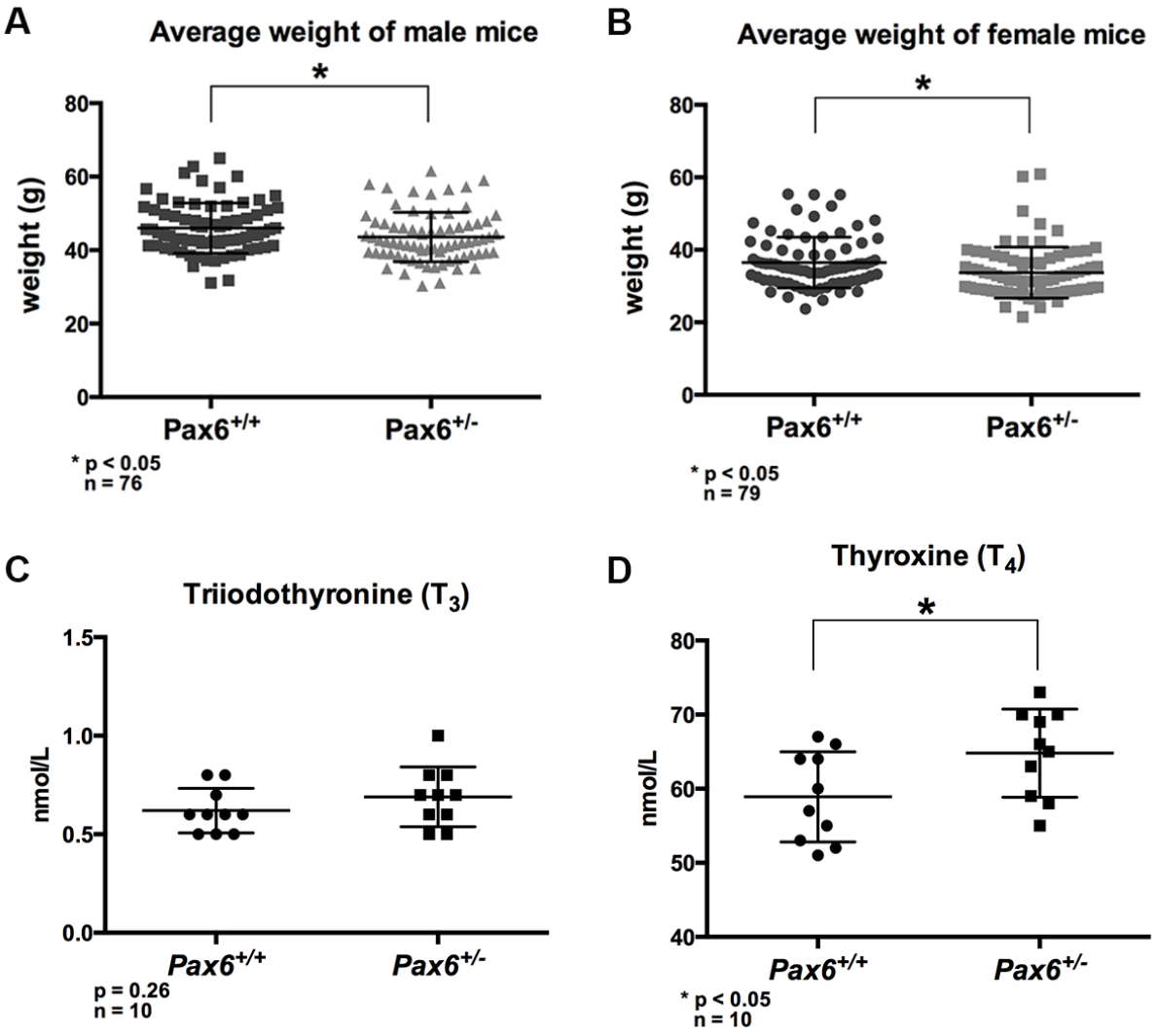
A
Average weight of male mice
*
weight (g)
80
60
40
20
0
Pax6+/+
Pax6+/-
* p < 0.05
n = 76
B
Average weight of female mice
*
weight (g)
80
60
40
20
0
Pax6+/+
Pax6+/-
* p < 0.05
n = 79
C
Triiodothyronine (T3)
nmol/L
1.5
1.0
0.5
0.0
Pax6+/+
Pax6+/-
p = 0.26
n = 10
D
Thyroxine (T4)
*
nmol/L
80
70
60
50
40
Pax6+/+
Pax6+/-
* p < 0.05
n = 10

Figure 2.6

Figure 2.6: Adult *Pax6*$^{+/-}$ mice weigh less than wild-type littermates and have increased T_4 levels in serum.

(A) In both male and (B) female mice, *Pax6*$^{+/-}$ mutant mice collectively weigh less than their wild-type littermates. Mice were 6 to 9 months of age. For each genotype 76 male mice and 79 female mice were weighed. Range: male wild-type, 31.8-65.0 g; male *Pax6*$^{+/-}$, 31.1-61.6 g; female wild-type, 23.7-55.0 g; female *Pax6*$^{+/-}$, 21.0-60.0 g (C) Concentration of total T_3 (TT_3) measured reveals no significant difference whereas (D) concentration of total T_4 (TT_4) is significantly higher in *Pax6*$^{+/-}$ mice than in wild-type littermates. Mice were 4 months of age. Serum was collected from 6 males and 4 females per genotype. Range TT3 (males + females): wild-type, 0.50-0.80 nmol/L; *Pax6*$^{+/-}$, 0.50-1.00 nmol/L. Range TT_4 (males+females): wild-type, 51.0-67.0 nmol/L; *Pax6*$^{+/-}$, 55.0-73.0 nmol/L. Data represented as mean ± 1 SD, *$p<0.05$

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CHAPTER 3

THE ROLE OF *Pax6* IN DEVELOPMENT OF ENTEROENDOCRINE CELLS

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Abstract

The small and large intestines of the verterbrate GI tract contain clusters of cells that release endocrine hormones, known as enteroendocrine cells. Two of these cell types, K cells and L cells, release gastric inhibitory peptide (GIP) and glucagon-like peptide 1 and 2 (GLP-1, GLP-2), respectively. GLP-1, especially, is important for making pancreatic islet β-cells competent to insulin sensing as well as regulating satiety, food intake, and body weight. *Pax6* is critical for development of L cells as well as transcription and synthesis of GLP-1. Patients with Aniridia have an increased incidence of obesity and diabetes that has traditionally been attributed to the reduction in *Pax6* in the pancreatic islets, but may also be a result of decreased GLP-1. This could presumably result in decreased satiety and increased food intake and body weight, possibly explaining the increased propensity for obesity in Aniridic patients. In exploratory experiments, we confirmed the presence of *Pax6* transcripts and protein in the small intestine, especially in the ileum, where L cells are most abundant. We also found that *Pax6* is co-expressed with GLP-1 expressing cells in the small intestine. Furthermore, the region where *Pax6* transcripts are most abundant, the ileum, also contains the most abundant PC1/3 mRNA as well. These findings provide preliminary evidence for further studies into the effects of a reduction in *Pax6* on the ability of GLP-1 to assist in regulation of glucose metabolism and weight maintenance, particularly in Aniridia patients. Furthermore, these potential findings could provide more therapeutic options for the treatment of type-1 diabetes mellitus.

Introduction

The vertebrate GI tract includes the esophagus, stomach, intestines, colon, as well as its derivatives, the liver, pancreas, and gallbladder. The main function of the GI tract is the breakdown and digestion of food, absorption of nutrients, and expelling the undigested parts of food. However, just as with the endocrine islets of Langerhans within the exocrine pancreas, there are a collection of cells of endocrine function found throughout the small and large intestine, and a portion of the stomach. While some of these cells, known as enteroendocrine cells, secrete hormones that aid in digestion to some extent, many have other functions that aid in homeostasis.

The small and large intestine is composed of three layers: endoderm, which will form the epithelial lining of the lumen, mesoderm, which will give rise to the smooth muscle, and ectoderm, which will form the enteric nervous system. Endodermal cells generate the lining of the GI tract while lateral plate mesoderm surrounds the lining to provide smooth muscle (1). Neural crest cells migrate in and settle throughout the tract to form the enteric nervous system (2,3). The gut develops from anterior and posterior invaginations at the end of the embryo, termed anterior intestinal porter (AIP) and caudal intestinal porter (CIP), respectively (4). These invaginations continue to elongate and fuse at the midline to form a straight tube. As the digestive tube meets mesenchymal cells, the latter instructs differentiation of the endoderm. Differentiation along the anterior to posterior axis of the tube will give rise to the formation of three regions: foregut (which will produce stomach, pharynx, and esophagus), midgut (which will give rise to small intestine) and hindgut (which will give rise to colon/large intestine). The mesenchymal cells further stabilize these boundaries.

The endodermal lining remains as a uniformed, undifferentiated layer of stratified cuboidal cells until midgestation, when epithelial-mesenchymal interactions direct endodermal differentiation (5–7). The villi of the small intestine begin to develop when the undifferentiated cells of the endoderm undergo a rostral to caudal transformation to a more columnar shape while proliferating epithelium that lie in between each forming villus will reshape downward to form crypts (8). These crypts and villi are found throughout the small intestine, giving it a substantial increase in surface area. Within the middle of these crypts are the stem cells found within the intestine. The mature small intestine contains a large number of stem cells, approximately 4-6 per crypt. The stem cells produce undifferentiated progenitors that will give rise to four different cell types: enterocytes, goblet cells, Paneth cells, and enteroendocrine cells (9). The large intestine is similar to the small, with the exception that the villi are a bit wider and shorter than the more thinner, longer villi characteristic of the small intestine and, in many vertebrates, these villi disappear in the mature large intestine (10,11). The large intestine contains all cell types found within the small intestine except Paneth cells.

Enterocytes are the most abundant cell type, making up the majority of cells within the gut. The main function of enterocytes is absorption and breakdown of nutrients for use by the body. Goblet cells, though significantly less than enterocytes, are the next most abundant intestinal cell type. Goblet cells secrete mucous, which acts as a protective barrier against contents of the intestines. Paneth cells and enteroendocrine cells make up the remaining cell types. Paneth cells function in antimicrobial defense and immunity while enteroendocrine cells release hormones that aid in movement of food products down the digestive tract and other functions through their interactions with the gut derivative organs as well as the brain.

Similar to the islets of Langerhans in the pancreas, enteroendocrine cells of the GI tract are not a whole gland but instead consist of a large number of endocrine hormone-secreting cells found throughout an organ that is otherwise devoid of endocrine functions. As part of the small and large intestine, enteroendocrine cells actively self-renew and differentiate throughout life, developing in the crypts and migrating to the top of the villi where, having reached their life-span, they are thought to undergo apoptosis (12). Although once thought to be derived from neuroectoderm, it is now widely accepted that enteroendocrine cells are of endodermal origin (13,14). Still, their differentiation is similar to that of neuronal cell differentiation, all developing from a common multipotent progenitor. Along with secreted proteins such as members of the *Wnt* and *Bmp* family as well as *Shh, Ihh* that regulate patterning of the intestines, there are three genes that are crucial for development of enteroendocrine cells: *Math1, Ngn3,* and Notch. *Math1* acts as a pro-endocrine differentiation gene, and the absence of its expression is a determining factor for secretory intestinal cell lineage, as *Math1* null mice do not develop goblet, Paneth, or enteroendocrine cell types in intestinal epithelium (15). The neuronal cell fate gene *Ngn3* is expressed in early in all enteroendocrine cells, beginning around E12.5 in mice, and its expression determines factor which secretory cells will become enteroendocrine cells. Mice with a $Ngn3^{-/-}$ mutation do not develop any endocrine cells within the small intestine (16). However, just as in the pancreas, *Ngn3* expression is down-regulated before terminal differentiation of endocrine cell types. Around the same time as *Ngn3* expression begins, Notch is expressed in secretory cells that are not fated to become endocrine cells but will become Paneth or goblet cells. Mutant mice with null mutation in Notch had increased *Ngn3* expression, leading to increased numbers of endocrine cells in the small and large intestines, confirming that Notch is

turned off in endocrine cells (17). Furthermore, endocrine cells in the gut act as activators of Notch expression in neighboring cells that are not fated for endocrine function.

There are eight main enteroendocrine cell types that secrete a variety of hormones: enterochromaffin cells (serotonin), I cells (cholecystokinin or CCK), K cells (gastric inhibitory peptide or GIP), L cells (glucagon-like peptide or GLP-1 and GLP-2), M cells (motilin), N cells (neurotensin), P cells (leptin), and S cells (secretin). Some of these hormones aid in digestion in some way, such as CCK stimulating digestion of fats and proteins or secretin and motilin regulating secretions of the stomach and pancreas and regulating peristalsis, respectively. Other hormones, specifically GIP and GLP-1, have an important role in homeostasis upon release. GIP acts on adipose cells to stimulate lipolysis and FFA synthesis as well as on the endocrine pancreas, along with GLP-1, to increase β-cell proliferation and survival as well as insulin synthesis and secretion (18). Furthermore, GLP-1 also acts to decrease glucagon secretion and increase glucose sensing as well as acting on the brain to increase nausea and feelings of satiety and decrease food intake and body weight (19–22). Finally, both GLP-1 and GIP help to make β-cells competent to glucose sensing, without which β-cells would not release insulin (18). GLP-2 has important maintenance effects on cell proliferation and apoptosis in the gut (23).

Pax6 is especially important for expression of GLP-1 and 2 as well as GIP (24). GLP-1 and GLP-2 in particular are produced by posttranslational processing of the proglucagon gene, a gene in which the role of *Pax6* in its transcription and regulation is well established (25,26). This processing is done with the aid of two members of the prohormone convertase (PC) family. Whereas in the pancreatic α-cells, glucagon is produced by the action of PC2 on proglucagon, in the enteroendocrine cells, GLP-1 and GLP-2 are produced by the actions of PC1/3 on the proglucagon gene (Figure 3.1). Studies have shown that *Pax6* binds to the G1 and G3 promoter

elements on the proglucagon gene to regulate its transcription and is also required for the synthesis of PC1/3 and PC2 (27,28). Indeed, *Pax6* haploinsufficient mice showed significant decreases in proglucagon mRNA and GLP-1 and GLP-2 expression in the small and large intestine. Furthermore, one study has shown that *Pax6* haploinsufficient mice have an increased metabolism as a result of down-regulation of GLP-1 (29). Additionally, *Pax6* null mutant mice also show a reduction in GIP expression. The co-expression of *Pax6* in GIP expressing cells further confirms its importance in GIP expression, along with *Pdx1*.

The actions of the hormones produced by K- and L-cells of the small and large intestine make them a provocative option for the treatment of diabetes and obesity due to their actions on β-cell neogenesis and proliferation in the pancreas and satiety in the brain. Indeed, the reduction in functional *PAX6* in Aniridic patients may also explain the complex nature of diabetes they experience and perhaps other symptoms not yet known. Exploratory experiments addressing how L and K cells respond in *Pax6* deficient mice may help answer questions about Aniridia as well as the possibility of using enteroendocrine cells in treating diabetes and obesity. To address this, we performed preliminary experiments using transgenic mice in an effort to determine our ability to detect *Pax6,* GLP-1, and PC1/3 expression in the small intestine as a possibility for future experiments addressing GLP-1 decrease as an explanation for Aniridic patients' severe obesity.

Materials and Methods

Animals

The transgenic mice were previously generated in the lab as described (31). All experiments involving mice were conducted in strict accordance with the National Institutes of

Health Guide for the Care and Use of Laboratory Animals and were performed with approval and oversight of the University of Georgia Institutional Animal Care and Use Committee.

Western Blot

Western analysis was performed on protein lysates prepared from whole eye, duodenum, jejunum, and ileum dissected from adult mice. Lysates were prepared by homogenization of tissues in RIPA buffer (10mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.1% SDS, 10% glycerol, 1mM PMSF, 1mM EGTA) in ice. Homogenized samples were centrifuged at 13,000 rpm for 5 minutes at 4°C, and the supernatant collected. Bradford Protein assay method was used to determine the protein concentration using bovine serum albumin (BSA) as the standard (Bio-Rad catalog 500-0006). 1-20μg of tissue protein to be analyzed were combined with equal parts 2X Laemmli sample buffer (Bio-Rad, catalog 161-0737) containing 10% β-mercaptoethanol, and then boiled for 15 minutes, loaded onto a reducing-denaturing SDS-polyacrylamide gel (10-12% resolving, 4% stacking), and one-dimensional electrophoresis was carried out for 4 hours at 75-100 volts in a Tris-glycine-SDS (Bio-Rad, catalog 161-0732) running buffer. Proteins were transferred to a 0.45μm nitrocellulose membrane (Bio-Rad, catalog 162-0115) in Tris-glycine transfer buffer containing 20% methanol cooled to 4°C. After transfer, the membrane was blocked overnight at 4°C using 5% non-fat dry milk (Bio-Rad, catalog 170-6404) in Tris-buffered saline pH 7.3, 0.1% Tween 20 (TBSTw) with agitation. Blots were incubated for 1 hour at room temperature with a 1:2000 dilution of a rabbit anti-*Pax6* primary antibody directed against the C-terminus of the protein (Covance, catalog PRB-278P) in blocking solution with gentle agitation. Following primary antibody incubation, membranes were rinsed several times in 1xTBSTw and incubated with goat anti-rabbit HRP-conjugated secondary antibody (1:20,000; Bio-Rad, catalog 179-5046; or 1:10,000; Santa Cruz, catalog sc-

2004) in 5% non-fat dried milk for 1 hour at room temperature with gentle agitation. After removal of the secondary antibody, the membranes were rinsed several times in TBSTw. For signal detection, Immuno-Star Western C Kit (BioRad catalog 170-5070) was used, followed by manual autoradiograph development.

Reverse Transcriptase PCR

Total RNA from whole eye, pituitary gland, brain, pancreas, stomach, duodenum, jejunum, ileum, and lung from an adult wild-type mouse was prepared using TRIzol reagent (ThermoFisher-Invitrogen , catalog 15596026) following the manufacturer's recommended conditions. Total RNA was then treated with the Turbo DNA-freeTM Kit (ThermoFisher-Ambion, catalog AM1907) following the standard protocol to remove potential DNA contamination. The treated RNA was then reverse transcribed (SuperScript Double-Stranded cDNA Synthesis Kit), and the resulting cDNA was amplified by PCR using a mouse *Pax6* primer pair designed by PrimerBank (*Pax6*: 7305369a1) and Roche (*proglucagon*: cat# 04685024001).

Immunofluorescence

To rapidly and uniformly preserve brain tissue after euthanization, adult mice were perfused with 1x phosphate-buffered saline (PBS) and 4% paraformaldehyde/PBS (PFA). Tissues obtained from dissected adult pituitary glands were preserved by immersion in 4% PFA at 4°C overnight, rinsed in 1x PBS, and then cryo-protected by taking through stepwise sucrose gradients (5%, 15%, 20%) followed by embedding in OCT medium and frozen at -20°C. Serial sections were cut using a rotary microtome at 8 µm, mounted onto slides (Superfrost/Plus; Fisher Scientific, Pittsburg, PA), and dried at 37°C overnight. Tissue sections used for indirect immunofluorescence were then incubated warm (37°C) PBS to remove OCT medium, then

rinsed in PBS and blocked for 30 minutes in a solution of 1% bovine serum albumin (BSA; Fisher Scientific, catalog BP1600-100), 5% normal donkey serum (Sigma-Aldrich, catalog D9663) in PBS. Overnight primary antibody incubation was performed in blocking solution at 4°C in a humidified chamber at a 1:1000 concentration. Primary antibody from Abcam (GLP-1: ab23472). After removal of the primary antibody, tissues were rinsed three times with PBS for 5 minutes each, blocked for 10 minutes, and incubated for 30 minutes at room temperature with a 1:1000 dilution of donkey anti-rabbit IgG (H+L) secondary antibody conjugated to Alexa Fluor® 647 (ThermoFisher-Invitrogen, catalog A-31573) in blocking solution. After removal of the secondary antibody, the tissue sections were rinsed several times with PBS, the nuclei were labeled with a 1:10,000 dilution of DAPI (4',6-Diamidino-2-phenylindole; Sigma, D9542) in PBS, and then coverslipped with EMS-Fluorogel.

Imaging of immunolabeled sections

Specific signals were visualized using either standard fluorescence microscopy using a Zeiss Axio Imager.D2 or laser scanning confocal microscopy using a Zeiss LSM 510 Meta Confocal Microscope and images acquired using Zeiss image acquisition software. 3 sets of animal intestines were analyzed for each stage and genotype; representative images are shown. N-values for each experiment are provided in the text and figure legends.

Quantitative Real time PCR

Total RNA from whole eye, duodenum, jejunum, and ileum from an adult wild-type mouse was prepared using TRIzol reagent (ThermoFisher-Invitrogen , catalog 15596026) following the manufacturer's recommended conditions. Total RNA was then treated with the Turbo DNA-freeTM Kit (ThermoFisher-Ambion, catalog AM1907) following the standard protocol to remove potential DNA contamination. The treated RNA was then reverse transcribed

(SuperScript Double-Stranded cDNA Synthesis Kit), and the resulting cDNA synthesis was conducted using the Thermoscientific kit. qRT-PCR was conducted using primer pairs designed by Roche (*Pax6*: cat# 04692136001, *Pcsk1*: 04688015001) and β-actin and α-tubulin as controls on Roche LightCycler thermocycler. All experiments were performed in triplicate.

Results

We previously generated BAC transgenic mice that were found to express EGFP in *Pax6* expression pattern in most tissues, with the exception of the diencephalon (31). Therefore, we decided to first use immunofluorescence using a GLP-1 antibody to determine if expression of GLP-1 overlapped with EGFP expression in the small intestine. We found that EGFP was indeed expressed in a subset of cell within the small intestine (Figure 3.2A, A'). We simultaneously labeled this same section with GLP-1. We found that GLP-1 was expressed in this region of the small intestine and, in most instances, this expression overlapped with EGFP expression (Figure 3.2B-C). This strongly confirmed that *Pax6* is co-expressed with GLP-1 expressing L-cells of the small intestine. Interestingly, there did appear to be some cells that expressed GLP-1 but did not express EGFP, and vice versa. This may indicate that our GLP-1 antibody cross-reacts with GLP-2 and the antibody is detecting both proglucagon derivatives. The EGFP expression that does not co-label GLP-1 may represent GIP expressing K cells. Although K cells are greater in density in the duodenum and L cells greater density than in the ileum, there is more overlap in the jejunum. We therefore decided to test for expression of *Pax6* and *proglucagon* within each region of the small intestine.

We extracted the small intestine and subdivided it into the three regions of the small intestine to test for the presence of *Pax6* and *proglucagon* transcripts. We also included portions of the lower stomach, pancreas, and brain. For *Pax6*, we used the eye as a positive control and

the lung as a negative control. For *Glp1*, we used the eye and stomach as a negative control. For *Pax6*, we found a strong band for the eye and brain as expected as well as a lighter band for the pituitary gland (Figure 3.3A). Confirming previous findings, we also found a strong band at the same size in the ileum, where *Pax6* expression is expected. Interestingly, we also found a light band in the duodenum and stomach, about the same intensity as that of the pancreas. The islets of Langerhans were not separated from the rest of the pancreas before RNA extraction, which may explain the lighter intensity of the band. The presence of a band for the stomach may indicate a role for *Pax6* in enterendocrine cells in the stomach. Also, *Pax6* has been shown to function along with *Pdx1* in regulating GIP expression in K cells, which are more abundant in the duodenum (24). This may explain the presence of the band for *Pax6* in this part of the small intestine. However, when we looked for the presence of PAX6 protein in the three regions of the small intestine, we found the expected 48/46 kD band only in the ileum but not in the duodenum (Figure 3.3C).

We also looked for *proglucagon* transcripts in these same regions. Although the absence of a band in the eye, stomach and lung indicate that the bands we did see represent *proglucagon*, we also saw presence of bands in all parts of the intestine, such as the duodenum, as well as brain and pituitary (Figure 3B). Since there is no known evidence of *proglucagon* in the duodenum or pituitary, more experiments would need to be done to confirm *proglucagon* transcripts. There does appear to be a much less intense band above the 200bp in the ileum and jejunum. It is possible that this band is more representative of *proglucagon* than the one below it. Still, additional experiments would need to be done to confirm.

We also wanted to determine copy number of *Pax6* in the small intestine as well. Although it did not appear to be highly expressed, we did find that *Pax6* is expressed in all three

regions of the small intestine, the majority of expression was found in the ileum, as expected (Figure 3.4B). Since PC1/3 is the enzyme that generates GLP-1 through posttranslational processing of proglucagon, we also wanted to look for its relative expression in the small intestine as well. Interestingly, there appeared to be nearly equal levels of PC1/3 expression throughout the small intestine, though very little based on relative expression levels (Figure 3.4A). There may be some role for PC1/3 in the processing of other enteroendocrine hormones in addition to *Glp1*. However, isolating the enteroendocrine cells from the much larger volume of the enterocytes would need to be done to yield more accurate results. Comparing expression levels to that of a positive control such as pancreatic islets of Langerhans may also yield more positive results and allow us to make conclusions about its expression in the small intestine.

Discussion

We decided to start this study of *Pax6* in GLP-1 positive cells as a possible explanation for the increased incidence of obesity and diabetes in patients with Aniridia. Several lines of evidence have shown that *Pax6* is necessary for development of GLP-1 in L cells of the small intestine as well as production of its processing enzyme PC1/3. The actions of GLP-1 on the brain in regulating hunger led us to hypothesize that since there is a reduction in *PAX6* in Aniridic patients, it may result in decreased feelings of satiety and an increase in food intake and weight. Additionally, the source of glucose intolerance in Aniridic patients is different than those without Aniridia, as it has been shown that the reduction in *PAX6* in a sample population in patients with Aniridia caused abnormal glucose metabolism as a result on the action of *PAX6* on PC1/3 for insulin processing (32). If *PAX6* also affects GLP-1 and PC1/3 in the gut, it may further exacerbate their symptoms due to less β-cell priming to respond to insulin.

We decided to first confirm that we could detect the presence of *Pax6* in the small intestines of our mice as well as expression of GLP-1 and PC1/3. While were able to detect the presence of *Pax6* transcripts and protein, our ability to detect GLP-1 was successful when using immunofluorescence but inconclusive when attempting to detect transcripts. Similarly, while we were able to detect PC1/3 in the gut, the relative expression levels were too low to be able to draw strong conclusions. However, while troubleshooting these experimental results, a collaborating lab studying GLP-1 levels in humans found no change compared to non-Aniridic test subjects (data unpublished). We, therefore, chose to discontinue this project. Surprisingly, in a separate, unknown study, when GLP-1 was measured in mice, $Pax6^{Sey/+}$ mice had dramatically reduced circulating levels of GLP-1, increased food intake, and secreted less insulin (29). These mutant mice were on an inbred strain, which may have impacted the results differently than what is expected in a human population.

Still, GLP-1 analogs are being used as a potential therapy for the treatment of type 2 diabetes (33). It has also been explored as a potential treatment for obesity as well (34). While there are adverse side effects associated with the GLP1 analogs, including diarrhea and nausea, it continues to be used as a positive therapy for treatment of diabetes and diabetes-associated obesity. Therefore, even though some Aniridic patients did not have a change in levels of GLP-1, it may still prove a useful form of treatment of their diabetes. Exploring the impact of a reduction and/or absence of *Pax6* on the role of GLP-1 in glucose tolerance and other parts of the endocrine system, either on an out-bred strain of mice or conditional knockout mice, remains an intriguing idea for continued studies.

Figures

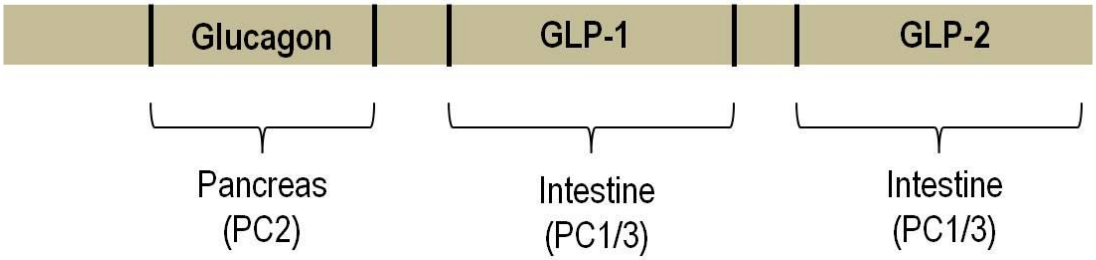

Figure 3.1

Figure 3.1: GLP-1 and GLP-2 are derivatives of the proglucagon gene
Glucagon-like peptides are produced by posttranslational modification of the proglucagon gene that is expressed in the L cells of the intestine. This modification is done through the actions of prohormone convertases (PC). Proglucagon is expressed in cells in the brain, GI tract, and pancreas. In the pancreatic islets of Langerhans, PC2 is responsible for the synthesis of glucagon while in the GI tract, PC1/3 is responsible for the production of GLP-1 and GLP-2.

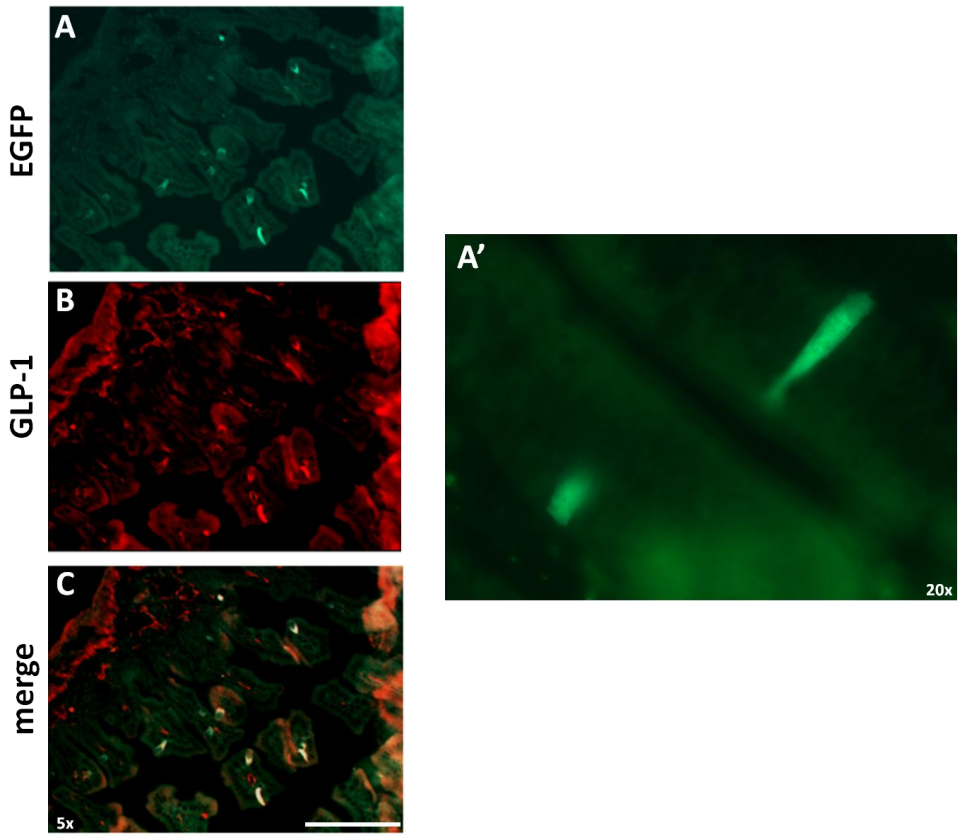

Figure 3.2

Figure 3.2 PAX6 is coexpressed with GLP-1 expressing intestinal L-cells in the small intestine

(A) EGFP in BAC transgenic mouse recapitulating *Pax6* expression pattern in enteroendocrine cells of small intestine **(A')** higher magnification of panel A showing EGFP expression in enteroendocrine cells **(B)** GLP-1 expression in small intestine, much of the expression in the same cells that express EGFP as evidenced by panel **(C),** which shows the merge of panel A and B. Scale bar = 100um, n = 3

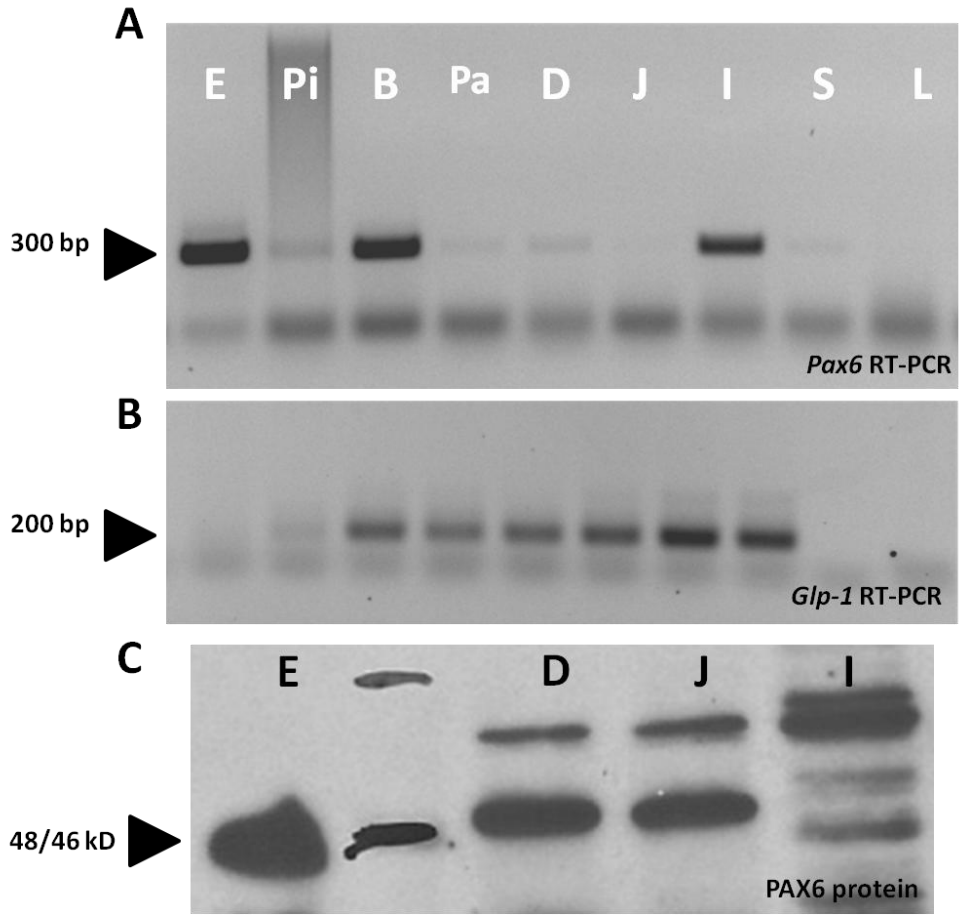
A
E
Pi
B
Pa
D
J
I
S
L
300 bp
Pax6 RT-PCR
B
200 bp
Glp-1 RT-PCR
C
E
D
J
I
48/46 kD
PAX6 protein

Figure 3.3

Figure 3.3: *Pax6* expression highest in ileum of small intestine
(A) RT-PCR shows 300bp band for *Pax6* transcripts present in ileum just as it is in the eye and brain positive controls while no band is seen in the lung negative control. **(B)** *Glp-1* transcripts also appear to be present in a number of tissues, including ileum, as expected, but absent in the eye and lung. More experiments needed to confirm *proglucagon* presence in the ileum since a band was seen in tissues where *proglucagon* is not expected, namely the duodenum. **(C)** Western blot shows presence of PAX6 protein in ileum by 46/48 kDa band indicative of canonical and 5(a) isoform of PAX6, but absent in duodenum and jejunum. Abbreviations: eye (E), pituitary (Pi), brain (B), pancreas (Pa), duodenum (D), jejunum (J), ileum (I), stomach (S), lung (L).

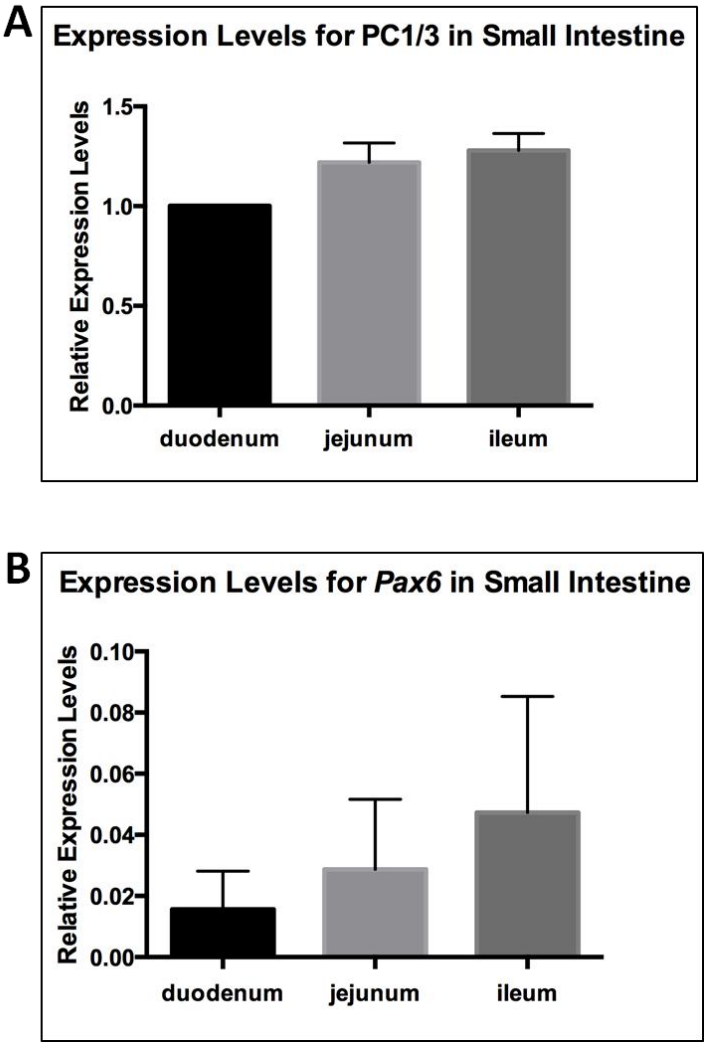
A
Expression Levels for PC1/3 in Small Intestine
Relative Expression Levels
1.5
1.0
0.5
0.0
duodenum
jejunum
ileum
B
Expression Levels for Pax6 in Small Intestine
Relative Expression Levels
0.10
0.08
0.06
0.04
0.02
0.00
duodenum
jejunum
ileum

Figure 3.4

Figure 3.4: Expression levels for *Pax6* and PC1/3 highest in ileum
(A) Expression of *Psck1,* or PC1/3, found in all parts of the small intestine, although relative expression was highest in ileum compared to jejunum and duodenum. **(B)** *Pax6* also expressed in all regions of the small intestine, although similar to PC1/3, expression levels of *Pax6* highest in ileum and, as expected, not very highly expressed in the duodenum.

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CHAPTER 4

NEUROANATOMICAL ABNORMALITIES IN *Pax6*-DEFICIENT MICE

To be submitted to *Brain Structure and Function.* Kenji K. Johnson and James D. Lauderdale.

Abstract

The *Pax6* gene encodes a highly conserved transcription factor that is expressed in the developing eye, brain, spinal cord and pancreas, and is required for various aspects of anatomical and functional development of the central nervous system. Heterozygous loss-of-function mutations of *PAX6* are causal for aniridia in humans. While the effects of *PAX6* mutations on ocular development have been well characterized in human and mouse, the implications of these mutations on brain structure remain poorly understood. Previous studies in humans have identified structural abnormalities in fiber tracts and subcortical structures in the brain including corpus callosum, anterior and posterior commissures, pineal gland and Probst bundles in persons with aniridia using magnetic resonance imaging (MRI). However, the high degree of variance in these studies means that a more consistent genetic background to be able to come to strong conclusions. Additionally, although the effects of a homozygous mutation in *Pax6* in mice have been well characterized, no studies to date have analyzed the effect of a heterozygous mutation on brain structure. The current study sought to determine whether structural brain abnormalities observed in patient case studies are present in the $Pax6^{Sey/+}$ mouse model of the disorder using histological measures of brain structure. We found that *Pax6* haploinsufficiency in mice resulted in hypoplastic white matter structures of the forebrain, decreased cellular density in the olfactory bulb and hippocampus, and expanded granule and molecular layers of the cerebellum. These results show consistencies with studies in humans and reveal new findings that may prove helpful in future studies of brain structure of patients with Aniridia.

Introduction

Pax6 expression in the developing embryo begins around E8.5 in the neural tube. As development proceeds, its expression becomes more restricted within the various brain regions. In the developing telencephalon, *Pax6* regulates dorsoventral patterning and in its absence, neurons in the neocortex fail to develop properly and ventral markers expand dorsally (1). *Pax6* also regulates regionalization and boundaries in the diencephalon as well as neuronal differentiation, leading to a reduction in cell number and volume in the thalamus in its complete absence and a loss of midbrain-pretectum boundary (2). In the cerebellum, a homozygous mutation in *Pax6* results in a reduction of certain regions, such as the pontine nuclei and aes, an absence in fissure formation, a disorganized granule cells and a broadened and disorganized external granule layer (3). Finally, *Pax6* also plays a role in axon guidance that is altered in its absence (4). Expression of *Pax6* in the adult brain becomes even more restricted, being found only in the subventricular zone, cerebellum, and slightly in the olfactory bulb and cortex (5). Much of what is known about the role of *Pax6* in the brain is a result of studies in homozygous mutant mice. However, *Pax6* is semidominant and a heterozygous mutation can also affect development of part of the central nervous system as well.

Aniridia is a congenital mutation characterized by iris hypoplasia and a host of additional ocular defects. More than 80% of all known cases of Aniridia are a result of *PAX6* haplo-insufficiency (6,7). While many studies have addressed the vision and diabetic complications associated with Aniridia, less is known about the effects of Aniridia on development on the brain. Some studies have begun to address defects in auditory processing and olfaction as well as decreased volume in anterior and posterior commissures and pineal gland (8–11). However, many of these studies have shown significant variation between patients, indicating that some of

the reported changes could be a result of modifier effects. Furthermore, no studies to date have addressed potential changes in the brain as a result of *Pax6* haplo-insufficiency at the molecular level.

We therefore decided to utilize histological analyses in the mouse model for Aniridia, *Small eye*, to look for changes in white or gray matter volume and/or cell proliferation that are consistent with what it seen in Aniridic patients or in homozygouse mutant mice. Potential structural and morphological changes we find in $Pax6^{+/-}$ mouse brains may indicate that the changes seen in human patients are consistent and also give some insight on other changes that may not have been found before. The results of this study revealed significant structural changes in both white and gray matter in regions where *Pax6* is expressed as well as areas where it is not expressed.

Materials and Methods

Animals

The mice used for this study were maintained as a $Pax6^{Sey\text{-}Neu/+}$ colony on a majority CD1 genetic background. Wild-type ($Pax6^{+/+}$) littermates were used as controls. The genotype of each animal was determined by PCR as previously described (12). All experiments involving mice were conducted in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were performed with approval and oversight of the University of Georgia Institutional Animal Care and Use Committee.

Sixteen mice (8 $Pax6^{Sey\text{-}Neu/+}$, 8 $Pax6^{+/+}$) 4-5 months (P122-153) of age, 50% male for each genotype were collected. One mouse (male, $PAX6^{Sey\text{-}Neu/+}$) died before histology could be collected, and one mouse of the same gender, age and genotype was used to replace this mouse

in the measures that were unable to be collected. The original 8 $Pax6^{+/+}$ and 7 $PAX6^{Sey\text{-}Neu/+}$ as well as the replacement animal are included in all brains that were collected for histology.

Tissue Collection and Histology

Mice were euthanized by CO_2 according to IACUC standards. To rapidly and uniformly preserve brain tissue after euthanization, adult mice were perfused with 1x phosphate-buffered saline (PBS) and 4% paraformaldehyde/PBS (PFA). Brains from these mice were extracted and preserved by immersion in 4% PFA at 4°C overnight, rinsed in 1x PBS, and then dehydrated stepwise through a graded series of 50, 70, 90, 96 and 100% ethanol, equilibrated with xylene, and embedded in paraffin using a Tissue Tek apparatus (Miles, Ekhart, USA). Serial sections were cut using a rotary microtome at 8 µm, mounted onto slides (Superfrost/Plus; Fisher Scientific, Pittsburg, PA), and dried at 37°C overnight. Tissue sections were deparaffinized by two rinses in xylene followed by rehydration in decreasing concentrations to 95% ethanol, then stained with a luxol fast blue solution overnight at 60°C. Slides were then washed in a 1% eosin and 1% cresyl violet solution separately, serially dehydrated in 95% and 100% ethanol and xylened followed by coverslip and sealed with mounting media.

Results

In mice with a homozygous mutation in *Pax6,* embryos fail to develop olfactory bulbs and have severe brain defects (13). Additionally, previous studies have demonstrated that the main olfactory bulb, but not the accessory olfactory bulb, exhibits age-dependent reduction in size and cell-types in *Pax6* haplo-insufficient mice (14,15). Therefore, we decided to first look for structural differences in the olfactory bulbs in $Pax6^{+/-}$ mice compared to wild-type. The most immediate difference in gross morphology was that the whole olfactory bulb was smaller in $Pax6^{+/-}$ mice compared to wild-type littermates, indicating a decrease in proliferation consistent

with roles previously established for *Pax6*. Interestingly, the external plexiform layer (EPL) of the *Pax6*$^{+/-}$ olfactory bulb is expanded, measuring approximately 370um between the glomeruler layer (GL) and molecular layer (ML) (Figure 4.1B) while that same region in wild-type littermates is approximately 295um (Figure 4.1A). There also appears to be fewer cells in the GCL layer of heterozygous mutant mice compared to the wild-type (Figure 4.1), again indicating a reduction in proliferation during development. This apparent decrease in cell number in the GCL may explain the reduced overall volume of the mutant olfactory bulb, despite the wider EPL. The reduction in cell proliferation that occurs in the forebrain in the complete absence of *Pax6* in homozygous mutants may also take place in heterozygous mutants and may explain the decreased density of the GCL in mutant mice.

Since several studies in Aniridic patients reported a change in interhemispheric commissures, we decided to look for white matter changes next. In the *Pax6*$^{+/-}$ brain, there appears to be a slight reduction in the anterior commissure (Figure 4.2B, D). This includes hypoplasia in the lateral most temporal limb of the AC compared to wild-type littermates (Figure 4.2A, C). As we continued to look more caudally, the anterior commissure appears to be displaced compared to its location in wild-type littermates (Figure 4.2C-D). Additionally, there appears to be a decrease in density of the olfactory limb of the anterior commissure in the heterozygous mutant brain compared to wild-type. This decrease in volume is consistent with some reports for patients with Aniridia. Other white matter tract differences in heterozygous mutant brains included an apparently broader lateral septal nuclei (Figure 4.2A-B), hypoplastic lateral olfactory tract (Figure 4.2A-B), reduced size of the ventral hippocampal commissure (Figure 4.2C-D), and reduction in volume of the habenula (Figure 4.2E-F).

We next decided to also look more closely at potential changes in the corpus callosum in *Pax6*$^{+/-}$ brains as well. We found that the volume of corpus callosum appears to be reduced in mutant brains, rostral-most (Figure 4.3A-D), mid-way through (Figure 4.3 G-H), and caudal-most (Figure 4.3 I-L). We also noticed that the parts of the anterior commissure that are visible were also reduced in *Pax6*$^{+/-}$ brains compared to wild-type littermates. The corpus callosum contains neural fibers that allow communication between the two hemispheres. Although we did not do cell counts to determine cell numbers, if there is a reduction in the number of cells, it could explain the reduction in volume as a result of less fibers in the tract.

Unexpectedly, we noticed changes in the dentate gyrus of the hippocampus in *Pax6*$^{+/-}$ brains. There appears to have been less proliferation in the ganglion cell layer in the mutant hippocampus, leading to reduced dorsal-ventral width of the GCL when compared to wild-type (Figure 4.4B, D). Indeed, in wildtype littermates, the width measured 32um compared to 25 um in *Pax6*$^{+/-}$. Additionally, there appears to be a reduced height within the polymorph layer of the *Pax6*$^{+/-}$ dentate gyrus compared to wildtype. When measured, the full height of the dentate gyrus (from the very top of the "point" straight down to the bottom) was approximately 140um in wild-type whereas in mutant mice it measured100um. Interestingly, when observing the length of the whole GCL of the dentate gyrus horizontally, it appears to be elongated in the *Pax6*$^{+/-}$ hippocampus compared to wild-type. These results were surprising because *Pax6* expression in the hippocampus is transient and not expressed in the adult brain. These results may be a result of defects in proliferation as a consequence of a reduction in *Pax6* expression earlier in development or an indirect effect changes in other brain regions, such as the pallium (cortex), where *Pax6* is expressed.

Pax6 expression is in the cerebellum is maintained in adult brains, albeit in a restricted pattern. Therefore, we expected that even a 50% reduction in functional protein may have an effect on cerebellum structure. When we looked at coronal sections, we found that the molecular layer of the eighth pyramidal region (PYRVIII) was expanded in mutant mice compared to wild-type (data not shown) as well as an expanded granular layer of the sixth declive region (DEC VI) in the *Pax6*$^{+/-}$ cerebellum compared to wild-type (Figure 4.5 E-F). No other structures within the hindbrain showed any major structural differences (Figure 4.5 A-D).

Discussion

In this study, we have attempted to histologically analyze adult *Pax6*$^{+/-}$ and wild-type mouse brains to determine if there are any structural changes due to a haplo-insufficiency in *Pax6*. We have found both white and gray matter differences in regions where *Pax6* expression is known to be expressed.

We found a decrease in cellularity and density in the EGL of *Pax6*$^{+/-}$ mice compared to wild-type littermates. Additionally, we found that the EPL also appeared to be expanded in mutant mice. The olfactory bulb develops as an extension of the telencephalon (16). Within the forebrain, *Pax6* has been shown to contribute to generating normal cell numbers, showing a decrease in cell proliferation in its absence (17). We believe that this change in cell density is due to the associated decrease in proliferation *Pax6* deficient forebrain. While these results reveal a structural change, based on evidence in Aniridic patients, these results likely mean *Pax6*$^{+/-}$ mice have compromised olfaction as well. There are several olfactory behavior protocols available that could be employed to test this hypothesis (18–20). Interestingly, the olfactory limb of the anterior commissure contains decussating fibers from the olfactory tracts of neurons within the olfactory bulb. This may at least partially explain the reduction in anterior

commissure volume that we found in *Pax6*$^{+/-}$ mice. Although no quantitative measures were taken on cellularity, the forebrain regions of heterozygous mutant mice appear to be less dense than wildtype littermates. This would explain the hypoplastic anterior commissure tract that we see in mutant mice. These fiber tracts, which connect the temporal lobes of the brain across the midline, contribute to memory, emotion, olfaction, and hearing, among other things. Behavior tests to determine if any functions of memory and hearing are compromised may further validate the changes we see if the anterior commissure of *Pax6*$^{+/-}$ mice.

We found that several other white matter tracts were hypoplastic in mutant mice as well. The hippocampal commissure, both the fimbriae and fornix, are reduced in *Pax6*$^{+/-}$ mice compared to wildtype. Since the hippocampal commissures are comprised of axons that carry information from one side of the hippocampus to the other so that both sides can communicate, it may be explained by reduced cell number and size of the hippocampal nuclei in *Pax6*$^{+/-}$ mice. Although some working memory deficits have been reported in Aniridic patients, no studies have been done to determine if there is a change in learning, memory, and spatial coding for which the hippocampus is controls. Tests in rats on contextual fear-conditioning may be of use in *Pax6*$^{+/-}$ mice as a behavior measure of the effects of a reduced and hypoplastic hippocampus (21).

The hypoplasia in the septal nuclei that we see in *Pax6*$^{+/-}$ mice is likely due to the gray matter differences in cell density that we see in other brain regions. The septal nuclei also receive connections from the olfactory bulb and hippocampus, in addition to numerous other regions. Furthermore, its projections to the lateral preoptic area, lateral hypothalamus, periventricular hypothalamus, and midline of the thalamus are all regions where *Pax6* is expressed during development and maintained in the adult. Although no definitive measures of cell numbers in the hypothalamus and thalamus were done, if there is a decrease in cell numbers

due to *Pax6* haploinsufficiency, any axonal connections that would have otherwise been made to those regions would be lost if there is a reduction in cell numbers. We believe this same logic can explain the hypoplasia in the corpus callosum. Corpus callosum fibers include commissural fibers which contains fibers from the cortex. Since there have been studies that show a change in gray matter within the cortex, it is plausible that there would be a reduction in the corresponding axons (Duan et al. 2013, our own unpublished data).

As expected, we found changes in the cerebellum as well. Both the molecular layer of the pyramidal region and the granular layer of the declive region were expanded in coronal sections. In sagittal sections, we also found that boundaries between fissures were also less defined and molecular regions appeared expanded as well (data not shown). The expansion of the granular layer of the declive region was surprising as *Pax6* is not known to control proliferation in the cerebellum (3). Based on evidence in *Pax6* homozygous mutant mice, we believe this expansion is most likely due to lack of proper migration in neurons that originate in this region but fail to migrate to their post-mitotic target sites. Since the molecular layer simply contains neuron cell bodies that are less tightly packed than the granular layer, this can likely explain the expansion of both the declive and pyramidal regions. The cerebellum is mainly responsible for motor control, although there is some thought that it may be involved in attention and language control as well. Still, the changes that we see structurally in the cerebellum of $Pax6^{+/-}$ mice do not correlate with any behavioral changes in the mice nor in people with Aniridia. However, if there is validity that the cerebellum is also associated with the cortex, it may add another layer to the pleiotropic effects of *Pax6* haploinsufficiency in the brain.

In conclusion, the results of this structural study of the effects of a heterozygous *Pax6* mutation in mice confirms some findings in human Aniridic patients and reveals new ones that

are note-worthy. These findings in mice may contribute to fully understanding how *Pax6* contributes to structural changes in the brain and how that affects behavior, particularly in humans and give more insight into the increasingly important role of a full dosage *Pax6* in central nervous system development.

Figures

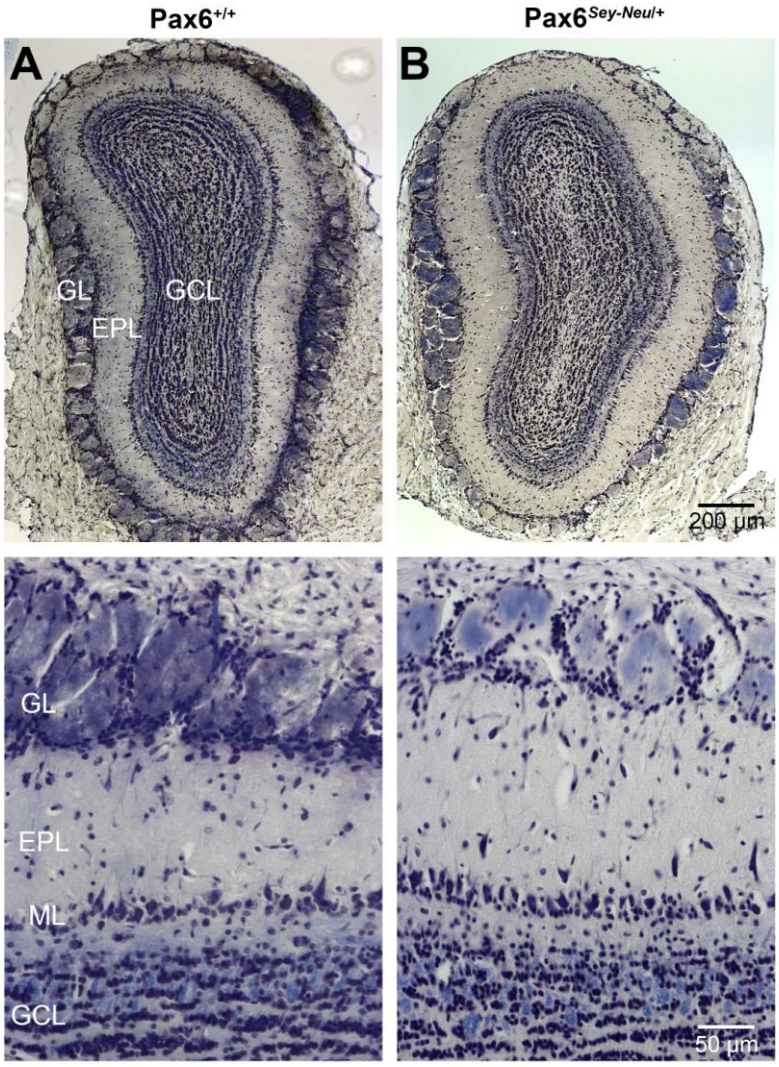
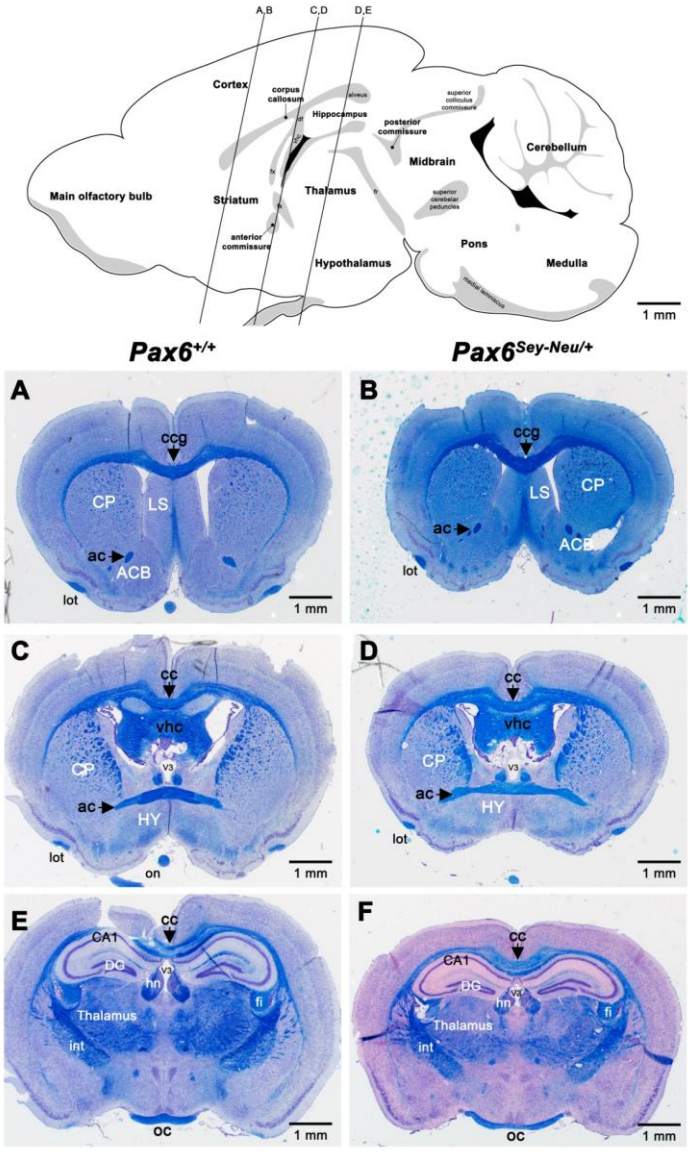


Figure 4.1

Figure 4.1 External Granule Layer (EGL) of olfactory bulb less dense in *Pax6*$^{Sey/+}$ mice
A. 5x coronal section of wildtype olfactory bulb with 20x below showing cellular density of external plexiform layer and molecular layer. **B**. 5x coronal section of *Pax6*$^{Sey/+}$ olfactory bulb showing decreased cellular density in granule cell layer and 20x below showing expanded external plexiform layer compared to wildtype. n=4.


A,B
C,D
D,E
Cortex
corpus callosum
Hippocampus
posterior commissure
Midbrain
Cerebellum
Main olfactory bulb
Striatum
Thalamus
anterior commissure
Hypothalamus
Pons
Medulla
1 mm
Pax6+/+
Pax6Sey-Neu/+
A
ccg
CP
LS
ac
ACB
lot
1 mm
B
ccg
LS
CP
ac
ACB
lot
1 mm
C
cc
vhc
CP
v3
ac
HY
lot
on
1 mm
D
cc
vhc
CP
v3
ac
HY
lot
1 mm
E
CA1
cc
DG
v3
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Thalamus
int
oc
1 mm
F
CA1
cc
DG
v3
hn
fi
Thalamus
int
oc
1 mm

Figure 4.2

Figure 4.2 Structural differences in white matter tracts in forebrain
A 5x coronal section through forebrain in wild-type brain **B** same region as (A) in *Pax6*$^{+/-}$ brain showing slightly hypoplastic anterior commissure, broader lateral septal nuclei, and hypoplastic lateral olfactory tract. **C** 5x coronal section through forebrain in wild-type brain caudal to (A) **D** same region as (C) in *Pax6*$^{+/-}$ brain revealing reduced volume in ventral hippocampal commissure and a seemingly displaced temporal limb of anterior commissure compared to wild-type and less dense olfactory limb of anterior commissure. **E** 5x coronal section through forebrain in wild-type caudal to (C) **F** same region as (E) in *Pax6*$^{+/-}$ showing regions a hypoplastic habenula compared to wild-type. n=3
Abbreviations: DG – dentate gyrus, V3 – third ventricle, lot – lateral olfactory tract, HY – hypothalamus, VHC – ventral hippocampal commissure, SN – septal nuclei, CC – corpus callosum, AC – anterior commissure, hn – habenula, int – internal capsule, cp – caudoputamen, ls – lateral septal nucleus, ccg – genu of corpus callosum, ACB – nucleus accumbens

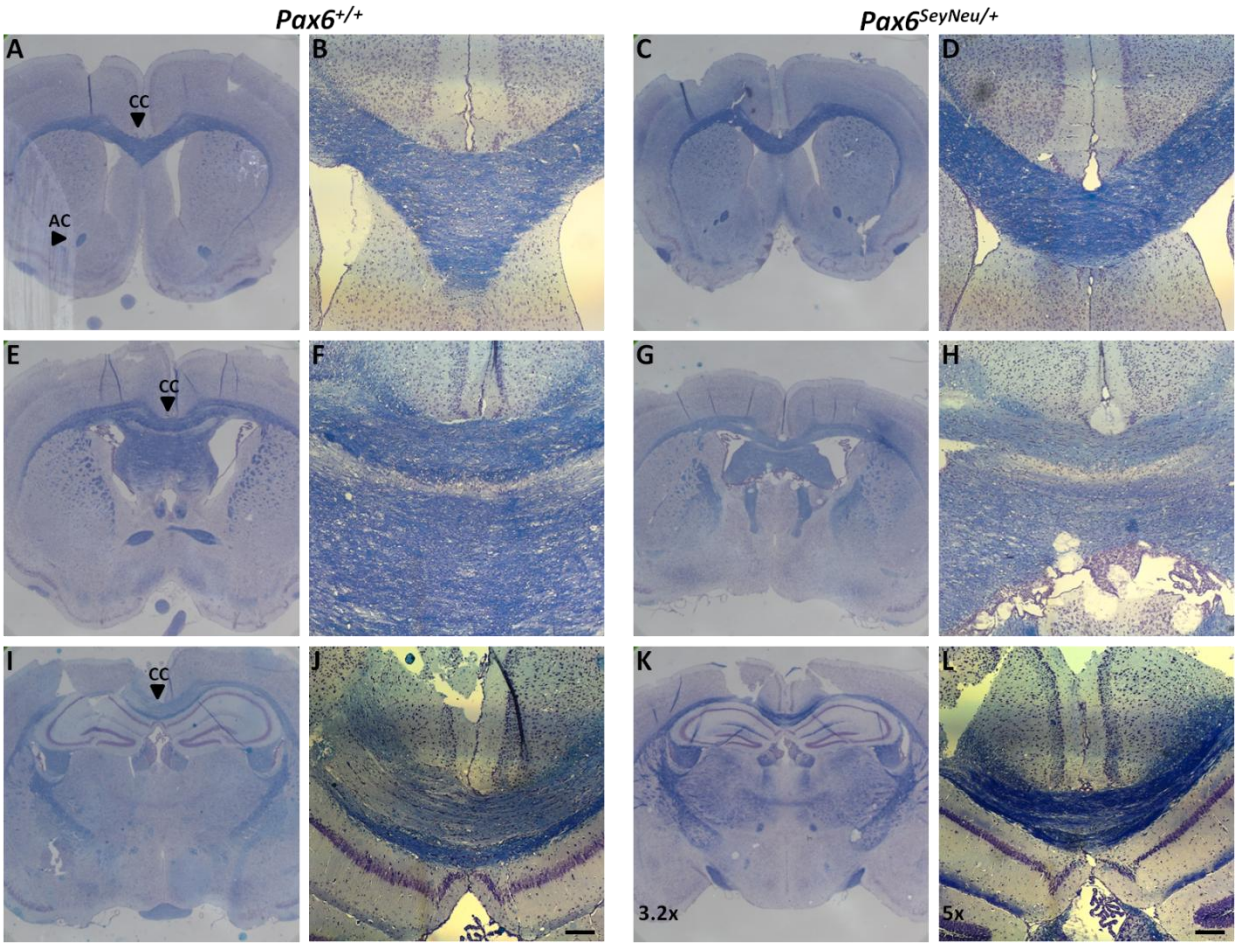

Figure 4.3

Figure 4.3 Reduced volume of corpus callosum reduced in brains of *Pax6*$^{+/-}$ mice
A,B – low and higher magnification of corpus callosum in wildtype mouse rostral-most, **E,F** – at mid-line, and **I,J** – caudal-most. **C,D** low and higher magnification of corpus callosum in *Pax6*$^{Sey/+}$ mouse showing reduced volume rostral-most, **G,H** – at midline, and **K,L** – caudal-most. Abbreviations: CC – corpus callosum, AC – anterior commissure. Scale bar = 100um, n = 3.

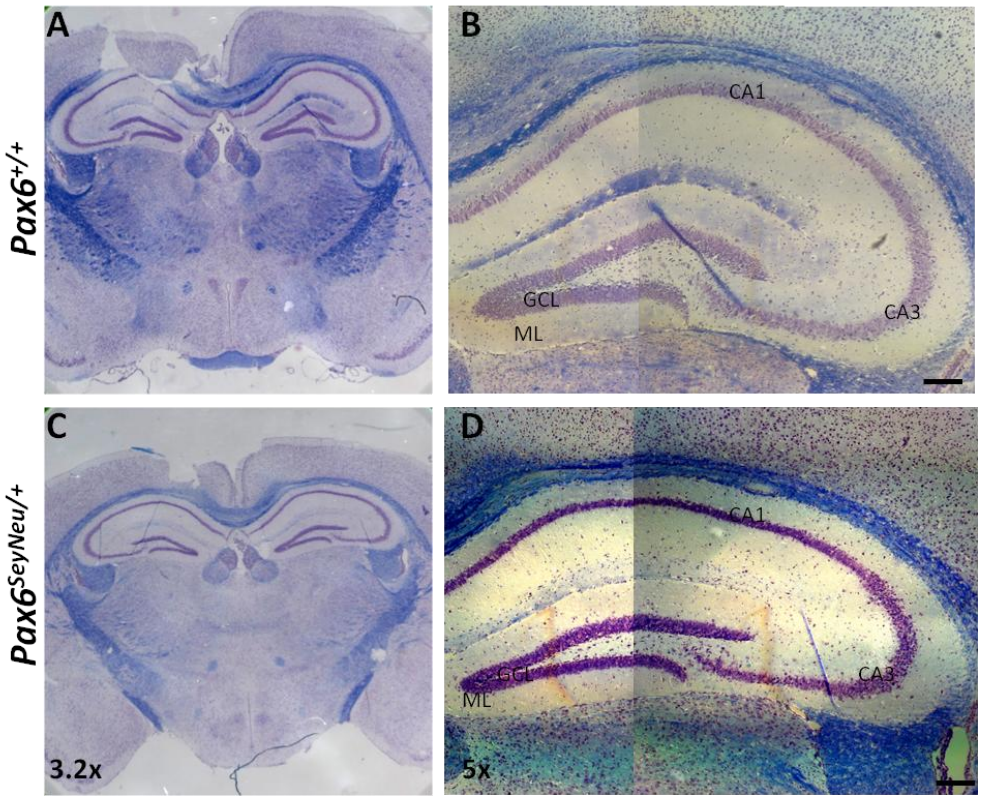

Figure 4.4

Figure 4.4 Structural differences in hippocampus of *Pax6*$^{+/-}$ mice
A. Coronal section of wild-type brain. **B**. Higher magnification of hippocampus of wild-type mouse showing normal architecture of dentate gyrus. **C**. Coronal section of *Pax6*$^{+/-}$ brain. **D**. Higher magnification of hippocampus of *Pax6*$^{+/-}$ showing decreased density in dentate gyrus as well as reduced polymorphic layer in dentate gyrus. Scale bar = 100um, n = 3.

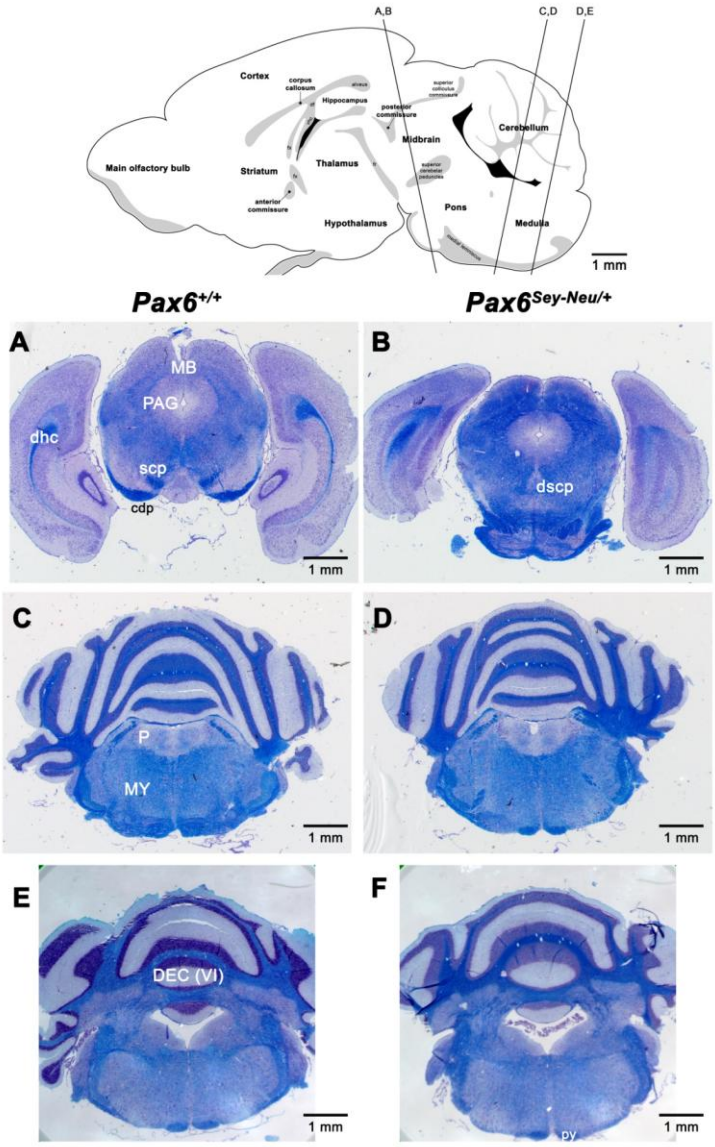
A,B
C,D
D,E
Cortex
corpus callosum
Hippocampus
posterior commissure
Midbrain
Cerebellum
Main olfactory bulb
Striatum
Thalamus
anterior commissure
Hypothalamus
Pons
Medulla
1 mm
Pax6+/+
Pax6Sey-Neu/+
A
MB
PAG
dhc
scp
cdp
1 mm
B
dscp
1 mm
C
P
MY
1 mm
D
1 mm
E
DEC (VI)
1 mm
F
py
1 mm

Figure 4.5

Figure 4.5 Expansion of gray matter regions in *Pax6*$^{+/-}$ cerebellum
A-B. 5x coronal section through midbrain/hindbrain region in wild-type (A) and *Pax6*$^{+/-}$ (B) showing similar architecture between both genotypes. **C-D** 5x coronal section through hindbrain in wild-type (C) and *Pax6*$^{+/-}$ (D) showing hindbrain, including pons and medulla. **E-F**. 5x coronal section through hindbrain sections showing over-proliferative and expanded declive region of *Pax6*$^{+/-}$ mice (F) compared to wild-type (E). Scale bar = 100um, n = 3.
Abbreviations: P – pons, MY – medulla, dscp – superior cerebellar peduncle decussation, dhc – dorsal hippocampal commissure, MB – midbrain, scp – superior cerebellar peduncle, PAG – periaqueductal gray.

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Chapter 5

Conclusions and Discussion

In this dissertation, we have sought to determine how a haplo-insufficiency in *Pax6* affects systems in which *Pax6* is crucial to its development. While this has mainly involved the pituitary gland and other parts of the endocrine system, we have also sought to determine if there were also any structural changes in the mature brain as well. Focusing our endocrine studies on the pituitary gland, we found that in a *Pax6* haplo-insufficient mouse, $Pax6^{Sey/+}$, there is a decrease in GH-producing cells and an increase in TSH-producing cells in the developing gland, the increase in TSH producing cells persisting in adults. This increase in TSH-producing cells results in an increase in the thyroid hormone T_4. Furthermore, we are the first to find that *Pax6* expression is maintained throughout the anterior lobe in the adult, indicating a maintenance role. We also found that the decrease in functional *Pax6* results in structural changes in the heterozygous mutant brain, including a less dense granule cell layer in the olfactory bulb and hippocampus, hypoplasia of the anterior commissure, hippocampal commissure, septal nuclei, and corpus callosum, and an expanded declive and pyramidal layer of the cerebellum. These findings are especially important since a mutation in one copy of the gene has traditionally been thought to only affect the eye. The data presented in this dissertation reveal that *Pax6* haplo-insufficiency profoundly affects a number of organs where it is important for development. While this opens new possibilities for scientific questions, it also means that patients affected by Aniridia could possibly suffer from a host of other complications that were previously undiagnosed. The data presented in this study only begin to answer more questions about the

role of *Pax6* in the pituitary gland and the hypothalamus. Future experiments to extend this study would also address how and why only T_4 is affected by the increase in TSH producing cells and not T_3, extend hormone measurements to TSH and TRH to see how the homeostasis is affected, and address the possible increase in metabolism in our mice, such as measuring food intake, fat mass, and body temperature. Finally, based on the results of this, it may be beneficial to measure some of these same hormones in our Aniridic patient population as well.

Still, even with this newfound evidence, there is still more that we do not fully understand. For example, our studies revealed no change in ACTH cell numbers, so we chose not to measure ACTH hormone levels. However, in their studies of the actions of *Pax6* on PC1/3, Wen et al found that, at least in people, there was a decrease in circulating levels of ACTH and α-MSH (1). A separate study also found that some Aniridic patients had a decrease in levels of ACTH (2). These changes are a result of how *Pax6* affects the enzymes that are responsible for producing the active form of prohormones, prohormone convertases. The role of *Pax6* in both PC1/3 and PC2 have been well described for their actions in the pancreas and intestines (3–5).

In addition to the pancreatic and incretin hormones, a number of hypothalamic and pituitary hormones are affected by prohormone convertases, including TRH, POMC, somatostatin, and arganine vasopressin (6–9). Furthermore, TRH, somatostatin, and arganine vasopressin neurons are found in the paraventricular region while POMC neurons are found in the arcuate nucleus, the same regions where *Pax6* is expressed in the developing and mature brain. If *Pax6* regulates PC2 and PC1/3 in the pancreas and intestines, it may also regulate its expression in the brain and pituitary gland as well. Several experiments measuring hormone levels of TRH, ACTH, SST, and AVP in $Pax6^{+/-}$ mice could begin to answer these questions.

Additional experiments would be needed to determine if there is a change in expression of PC1/3 and/or PC2 in the hypothalamus and pituitary gland and confirm their regulation by *Pax6* to begin to answer some of these questions. Although there are certainly numerous ways in which a *Pax6* deficiency can affect homeostasis, a deficiency in these processing hormones could explain the obesity experienced by patients. At least two studies have provided evidence for a link between these prohormone convertase enzymes and an increased propensity for obesity as well as affects on the gonads and adrenal glands (10,11). If there is a deficiency in production of the active TRH, it could further compound the effects of the increased number of thyrotropes in the *Pax6* deficient pituitary gland. These prohormone convertase enzymes are also expressed in the hippocampus and cerebral cortex (12,13). As shown by our structural studies in this dissertation and other studies using fMRI (data unpublished), a deficiency in *Pax6* changes the structure of these regions. The decrease in functional PAX6 may also impact production of prohormone convertase enzymes in these regions as well. Although their expression in the cortex and hippocampus may not impact hormones, if affected by *Pax6* it may also impact the secretory peptides that they process as well. Additionally, our findings on the structural and morphological changes in the brain of $Pax6^{+/-}$ mice indicate that if the hypothalamic region is affected, it could impact hypothalamic hormones. If so, this could further impact the already complex endocrine system, particularly in the case of GnRH and TRH. Both of the neurons originate in the olfactory and optic region, respectively (14,15). Since the structure of these regions is impacted in $Pax6^{+/-}$ mouse brains, it is plausible that these hypothalamic neurons would also be affected. Experiments exploring expression pattern and measuring their hormone levels, as mentioned earlier, could quickly answer these questions.

It is interesting and compelling to understand how a haplo-insufficiency in *Pax6* affects the endocrine system and how it might affect Aniridic patients, but these studies still leave several questions about the mechanism by which *Pax6* impacts development of pituitary and hypothalamic cells. Studies in the pancreas and enteroendocrine cells have revealed much about the role of *Pax6* in the endocrine system, such as how it not only regulates the development and maintenance of pancreatic islets and some enteroendocrine cells, but also the genes that are expressed there and required for their proper functioning (3,16–18). Several genes that are expressed in the developing pituitary gland and hypothalamus, such as *Six3/6, Nkx2.1/2.2, NeuroD, Isl1, and Lhx3/4* are also known to interact with *Pax6* in the developing eye, spinal cord, and/or pancreas. Studies that addressed how *Pax6* interacts with these transcription factors in the neuroendocrine organs could shed new light on how *Pax6* regulates hormone producing cells in the brain and pituitary gland. For example, in the absence of a full dosage of *Pax6,* somatotropes and lactotropes are decreased in number while thyrotropes increase. While it is suggested that *Pax6* represses the actions of BMP2 in the pituitary gland, thus causing the expansion of ventral cell types at the expense of dorsal cell types, studies that directly addressed this putative mechanism would definitively answer these mechanistic questions. Still, *Pax6* is only confined to the dorsal part of the anterior lobe through E12.5 (19). Thereafter, it is expressed in all hormone cell types throughout the anterior lobe. As mentioned earlier, this indicates a maintenance role for *Pax6.* Mechanistic assays, perhaps using cell culture or ChIP assays, may reveal how *Pax6* interacts with other genes to maintain expression of the anterior lobe cell types. It might also be interesting to see how the system responds in conditional inactivation of *Pax6* only in the developing pituitary such as what was done to see the effects in the pancreas (20). If the homozygous mutant pups were able to survive beyond the first day of

birth the numbers somatotropes may be restored such as is done in the heterozygous condition. However, since the cell numbers change even more drastically in homozygous mutants, it is possible that conditional mutant pups would have growth defects as a result of the decreased GH-producing cells and/or severe hypermetabolism as a result of the increased TSH-producing cells and unable to gain the necessary weight for survival.

The endocrine system is an increasingly complex system. The role of *Pax6* in regulating development of the endocrine system, particularly the neuroendocrine portion, is only beginning to be studied in more detail. The work presented in this dissertation highlights the dosage sensitivity of *Pax6* and the necessity in gaining a better understanding of how these changes affect Aniridic patients since it affects hormone producing cell numbers, hormone levels, and presumably homeostasis. Placing more emphasis on studying the role of *Pax6* in the hypothalamic and pituitary portions of the endocrine system may finally begin to reveal causative factors for the systemic symptoms experienced by Aniridic patients.

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