NEUROANATOMICAL AND SENSORY PROCESSING ABNORMALITIES IN ANIRIDIA: PAX6 MUTATIONS AND THEIR IMPLICATIONS IN THE BRAIN

by

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(Under the Direction of James D. Lauderdale and Brett A. Clementz)

ABSTRACT

The paired-box 6 (*PAX6*) gene encodes a highly conserved transcription factor that is essential for proper development of the eye and brain. Heterozygous loss-of-function mutations in *PAX6* are causal for a condition known as aniridia in humans and the *Small eye* phenotype in mice. Aniridia is a predominantly characterized by iris hypoplasia and other ocular abnormalities, but recent evidence of neuroanatomical, sensory, and cognitive impairments in this population has emerged, indicating brain-related phenotypes as a prevalent feature of the disorder. Determining the neurophysiological origins of brain-related phenotypes in this disorder presents a substantial test, as the majority of extra-ocular traits in aniridia demonstrate a high degree of heterogeneity. Similarly, the molecular mutations of *PAX6* that are causal for aniridia are highly variable. These challenges demonstrate that a single line of investigation is insufficient for understanding aniridia as a whole. The work presented here implemented three approaches towards an integrative understanding of this condition: (i) molecular genetic analysis of human *PAX6* mutations to investigate the spectrum of molecular changes associated with the disorder, (ii) structural magnetic resonance imaging (MRI) of

a rodent model of aniridia to determine the neuroanatomical consequences of *PAX6* mutations which are conserved across species and attributable to *PAX6* deficiency, and (iii) an auditory paradigm administered to humans with aniridia and measured by electroencephalography (EEG) to examine the fine temporal and spectral dynamics of auditory processing dysfunction in this disorder. Together, these experiments contribute to our understanding of aniridia from molecular, anatomical, and functional perspectives, and provide a framework for investigating the link from genetic mutations to complex neural abnormalities.

INDEX WORDS: *Small eye* mouse, magnetic resonance imaging, electroencephalography, auditory processing

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DEDICATION

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1:

INTRODUCTION AND LITERATURE REVIEW

1.1 PAX6: Gene structure, products, functional mutations and associated disorders

1.1.1 Gene structure and products

The paired-box 6 (PAX6) gene is an approximately 22kb region located on chromosome 11p13 in humans and chromosome 2 in mice and encodes a homeobox-like transcription factor (Siracusa & Abbott, 1992; Walther & Gruss, 1991). The PAX6 sequence is highly evolutionarily conserved from *Drosophila* to mice and humans, and also demonstrates high conservation of regulatory and enhancer sequences in vertebrates (Glaser, Walton, & Maas, 1992; Kleinjan, Seawright, Childs, & van Heyningen, 2004; Morgan, 2004; Plessy, Dickmeis, Chalmel, & Strähle, 2005; Quiring, Walldorf, Kloter, & Gehring, 1994). PAX6 includes 16 exons (exons 4 through 13 encompass the coding region) and 3 promoter regions (P0, P1 and Pα), which give rise to at least 3 isoforms of the PAX6 protein, referred to as canonical PAX6, PAX6(5a), and PAX6(ΔPD) (Kammandel et al., 1999). Canonical PAX6 is composed of four functional domains: two DNA binding domains (a paired domain and a homeodomain) which are joined by a linker domain, followed by a proline-serine-threonine (PST) rich transactivation domain (Epstein et al., 1994; Tang, Singh, & Saunders, 1998; H. E. Xu et al., 1999). The PAX6(5a) isoform has the same conformation as canonical PAX6 plus an alternatively spliced exon 5a, resulting in an insertion in the paired domain which alters its DNA binding activity (Chauhan, 2004). The final isoform, PAX6(ΔPD) is a product of one of three alternative

translational start sites found in the linker domain sequence. Proteins of this kind are collectively referred to as the paired-less isoforms of PAX6 because they lack the paired domain present in canonical PAX6 and PAX6(5a) (Kim & Lauderdale, 2006). Because these isoforms are a result of different transcriptional and post-transcriptional regulation and possess distinctive DNA binding domain conformations, it follows that they have unique spatiotemporal expression and regulatory profiles during development and throughout the organism's lifespan (Anderson, Hedlund, & Carpenter, 2002; Chauhan, 2004; Kammandel et al., 1999; Sasamoto et al., 2016). However, the discrete differences in isoform expression profiles and functions remain under investigation.

1.1.2 Functional mutations/mutational models

Multiple mouse strains harboring functional Pax6 mutations have been generated to study its role in the brain, as well as to investigate the effects of various mutations in the context of human PAX6-mediated disorders. Four common alleles of Pax6 are semidominant mutations which are comparable to various human mutation cases. The *Dickie's Small eye* allele (*Sey-Dey*) is a spontaneous large deletion on chromosome 2 which encompasses Pax6 and the neighboring Wt1 locus, originally discovered in 1964 by Dr. Margaret Dickie and later phenotypically characterized (Theiler, Varnum, & Stevens, 1979). Another large chromosomal deletion of Pax6 is seen in the Sey^{H} allele, which was radiation induced (Hill et al., 1991; Lyon & Searle, 1989). The Sey^{H} and *Sey-Dey* alleles present the most devastating phenotypic outcomes compared to other Pax6 mutation strains: in both cases homozygotes die shortly after implantation, while heterozygotes have reduced overall body size and either lack eyes entirely, or display a severe reduction in the size of eye tissue (Hill et al., 1991; Theiler et al., 1979). The

phenotypic severity of these alleles is ascribed to the large size of the deletion and additional loci affected.

The spontaneously derived *Small eye* (*Sey*) allele consists of a single point mutation, specifically a G>T transversion at c.194 which causes a premature termination codon (PTC) in exon 5 (Hill et al., 1991; Hogan et al., 1986; Roberts, 1967). Finally, the *Small eye Neuherberg* allele (Sey^{Neu}) is also a single point mutation which was established by an ENU (*N*-ethyl-*N*-nitrosourea) mutagenesis experiment (Favor, Neuhäuser-Klaus, & Ehling, 1988). This mutation is a G>T transversion at the +1 position of intron 10, and causes a splice junction disruption which leads to an additional 116 nucleotide extension and ultimately a PTC found in the intronic sequence (Hill et al., 1991). Both the Sey^{H} and the Sey^{Neu} homozygous mutants do not survive past postnatal day 1 (P1) and demonstrate severe craniofacial and nasal underdevelopment, while heterozygous mutants display the characteristic reduced eye size (Grindley, Davidson, & Hill, 1995; Hill et al., 1991; Hogan, Hirst, Horsburgh, & Hetherington, 1988). In addition to these mutation models, transgenic lines also exist, including a conditional allele of Pax6 ($Pax6^{flox}$) and a LacZ Pax6 knock-out reporter which allows examination of Pax6 specific developmental functions and expression patterns (R Ashery-Padan, Marquardt, Zhou, & Gruss, 2000; Ruth Ashery-Padan, 2002; St-Onge, Sosa-Pineda, Chowdhury, Mansouri, & Gruss, 1997). The latter has been especially useful for elucidating the spatial and temporal specific functions of Pax6 *in vivo*. However, the first four mutations described are most comparable to those which are associated with diseases in humans.

1.1.3 Human PAX6 mutations and PAX6-mediated disorders

In humans, as in mice, homozygous loss-of-function mutations of PAX6 are embryonic lethal. Heterozygous mutations in PAX6 are implicated in several human disorders. Most

prominently, loss-of-function mutations in PAX6 in humans are causal for a condition known as aniridia, which can be classified as syndromic or isolated. Syndromic aniridia, also known as Wilms tumor-aniridia-genital anomalies-retardation (WAGR) syndrome, is caused by large deletions which encompass the PAX6 and WT1 loci (Fischbach, Trout, Lewis, Luis, & Sika, 2005; Hingorani & Moore, 2013; S. Xu et al., 2008). Individuals with WAGR syndrome are at high risk for developing Wilms tumor, have severe ocular phenotypes and are most accurately modeled (genotypically and phenotypically) by the *Sey-Dey* and Sey^{H} alleles in mouse models (Glaser, Lane, & Housman, 1990). Isolated aniridia is characterized by a lack or hypoplasia of the iris, and the majority of cases (≥80%) are caused by loss-of-function heterozygous mutations of PAX6 or deletions of associated regulatory regions of the gene (Hanson et al., 1993). This type of aniridia occurs at a rate of approximately 1.2 in 100,000 live births (Grønskov et al., 2014). Isolated aniridia is best modeled by Sey^{H} and Sey^{Neu} mouse alleles (Jordan et al., 1992). The less frequent cases of aniridia for which a mutation in PAX6 cannot be identified are often associated with mutations in FOXC1, PITX2 or PITX3 (Ito et al., 2009; Khan, Aldahmesh, & Al-Amri, 2008; Law, Sami, Piri, Coleman, & Caprioli, 2011; Semina et al., 1998; Tümer & Bach-Holm, 2009).

A large number of PAX6 loss-of-function mutations causal for this disorder are nonsense substitutions which lead to PTCs. In these cases, there is a high prevalence of C>T transitions at CpG dinucleotide "hotspots" (Hanson et al., 1993). Additionally, approximately 30% of PAX6 mutations in aniridia are caused by small insertions and/or deletions, and 20% are caused by mutations which disrupt mRNA splicing (Hanson et al., 1993; Prosser & van Heyningen, 1998). Mutations are found throughout the coding region of the gene, with a particular preponderance of mutations affecting the portion of the PAX6 coding sequence responsible for the paired domain.

Run-on mutations caused by missense substitutions in exon 13 have been identified in a few cases of aniridia, and are generally associated with a more severe phenotype because of the likelihood that a dominant negative protein is produced from the mutant allele (Singh, Chao, Mishra, Davies, & Saunders, 2001). However, much controversy exists in the field with regard to whether PTC-causing mutations lead to haploinsufficiency or idiosyncratic truncated protein products. Some researchers have proposed that dominant negative PAX6 proteins are present in persons with mutations in various locations in the coding region of the gene (Singh, Tang, Lee, & Saunders, 1998; Tzoulaki, White, & Hanson, 2005). However, others hold that these mutations represent a class of haploinsufficient cases because the nonsense-mediated mRNA decay surveillance pathway degrades all transcripts with a PTC before they can be translated (Chang, Imam, & Wilkinson, 2007; Khajavi, Inoue, & Lupski, 2006; Kokotas & Petersen, 2010). These molecular phenomena are critical in considering the individual phenotypic manifestation of the disease, particularly in terms of diagnosis and treatment.

In addition to the iris, aniridia is also frequently associated with foveal hypoplasia and nystagmus presenting early in development, as well as progressive ocular defects including glaucoma, cataracts, corneal opacification and vascularization (Netland, Scott, Boyle, & Lauderdale, 2011; Peter et al., 2013). The variation in these associated characteristics is considered to be indicative of differential genotype-phenotype manifestations, though this has not been conclusively determined (Dharmaraj et al., 2003; Dubey, Mahalaxmi, Vijayalakshmi, & Sundaresan, 2015; Tzoulaki et al., 2005). Besides aniridia, mutations of PAX6 have also been associated with other disorders affecting the eye, endocrine or nervous system. PAX6 mutations have been implicated in diabetes, congenital cataracts, optic nerve malformation, autosomal dominant keratitis (ADK) and Peter's anomaly (R Ashery-Padan et al., 2004; Noriyuki Azuma et

al., 2003; Mirzayans, Pearce, MacDonald, & Walter, 1995; Nishi et al., 2005; Prosser & van Heyningen, 1998). Missense mutations of PAX6 have been associated with autism spectrum disorder, isolated foveal hypoplasia and other independent ocular anomalies (N Azuma et al., 1999; N Azuma, Nishina, Yanagisawa, Okuyama, & Yamada, 1996; Maekawa et al., 2009), and these disorders have also demonstrated comorbidity with aniridia diagnoses (Davis et al., 2008; Kokotas & Petersen, 2010). Many of the phenotypes in PAX6-mediated disorders can be explained by the developmental role of PAX6 as a transcriptional regulator, which is of particular interest to the current body of work in the context of neurodevelopment.

1.2 The role of PAX6 in the brain

PAX6 is expressed at high levels during mammalian embryonic development in the eye, brain, spinal cord and pancreas (Kim & Lauderdale, 2006). The critical regulatory functions of PAX6 (and the *Drosophila* homolog of the gene "eyeless") in the development and maintenance of brain and eye tissues have been extensively characterized in mouse models and in *Drosophila* (R Ashery-Padan & Gruss, 2001; Grindley et al., 1995; Halder, Callaerts, & Gehring, 1995; Jang, 2003). The functional role of PAX6 in neurodevelopment has been studied through examining spatiotemporal expression patterns as well as assessing functional necessity and sufficiency using genetic models.

1.2.1 Spatiotemporal expression of PAX6 in development

In the developing brain, PAX6 expression begins as early as mouse embryonic day 8 (E8) in the ventricular zone of the basal and medial neural tube (Walther & Gruss, 1991). As development progresses, PAX6 is expressed in the mitotic germinal zone, followed by the telencephalon, diencephalon, mesencephalon (though not the roof of the mesencephalon) and hindbrain with expression boundaries corresponding to those of developing brain regions (A

Stoykova & Gruss, 1994; Walther & Gruss, 1991). Specifically, at E11, PAX6 demonstrates high levels of expression in the neuroepithelia of the ventricular zones in the forebrain and hindbrain, as well as the mesencephalic reticular formation and floor of the midbrain (Duan, Fu, Paxinos, & Watson, 2013). In the dorsal forebrain at E13, PAX6 is expressed in the telencephalon in a rostral to caudal gradient and demonstrates a boundary at the posterior commissure, which is the anatomical border between the last prosencephalic neuromere, the synecephalon and the mesencephalic rostral neuromere (A Stoykova & Gruss, 1994). At E12, E14 and postnatal day 1 (P1) PAX6 expression is restricted to the developing forebrain and hindbrain and is absent throughout the entire midbrain (Duan et al., 2013). The majority of PAX6 expressing cells are still distinguishable in adulthood, though the level of expression weakens significantly (Duan et al., 2013). The boundaries of PAX6 expression at various stages of embryonic development and through young adulthood indicate a role of PAX6 in regionalization, particularly in the forebrain. Further, these findings suggest a role of PAX6 in specification of neural progenitor fates in these territories (Duan et al., 2013; Manuel & Price, 2005; A Stoykova & Gruss, 1994). PAX6 also shows co-localization with differentiated tyrosine-hydroxylase (TH) neurons throughout development, implicating it in the specification of subpopulations of granule cell populations and dopaminergic neuronal fates (Duan et al., 2013; Kohwi, Osumi, Rubenstein, & Alvarez-Buylla, 2005; A Stoykova & Gruss, 1994).

1.2.2 Functional roles of PAX6 in development: from cells to circuits

While expression studies of PAX6 provided correlative evidence for a role of this transcription factor in regionalization, boundary formation and specification of neuronal subtypes, gain- and loss-of-function experiments using mutant and transgenic models have illuminated many of the functional mechanisms by which the Pax6 protein regulates

developmental processes and specification of neuronal cell fates throughout the brain. In heterozygous *Sey* mice, the olfactory bulb shows a significant reduction in volume which becomes more pronounced from P42 to P70, and co-localization of PAX6 with TH and gamma-aminobutyric acid (GABA) but not calretinin or calbindin expressing cells (Dellovade, Pfaff, & Schwanzel-Fukuda, 1998). When green fluorescent protein (GFP) labelled forebrain progenitor cells from homozygous *Sey* embryos were transplanted into wild-type mice, these cells were capable of migrating to the olfactory bulb, but failed to differentiate into dopaminergic perigloumlar or superficial granule cells (Kohwi et al., 2005). These studies provide evidence for a cell autonomous function of PAX6 in specifying dopaminergic cell differentiation, and possibly in GABA-dependent interneuron differentiation.

In the ventricular zone of the dorsal telencephalon, PAX6 shows high expression in the apical progenitor cells which are responsible for generation of cortical projection neurons and is involved in radial glia differentiation in early corticogenesis (Englund et al., 2005; Gotz, Stoykova, & Gruss, 1998; Heins et al., 2002). For example, homozygous *Sey* mice display a reduction in the size of the cortical plate and an expansion of the proliferative zone (Schmahl, Knoedlseder, Favor, & Davidson, 1993; A Stoykova, Fritsch, Walther, & Gruss, 1996). These investigations revealed widespread disruptions in dorso-ventral patterning in the telencephalon and significant cortical forebrain patterning defects in these mice (Manuel & Price, 2005; A Stoykova et al., 1996; Anastassia Stoykova, Treichel, Hallonet, & Gruss, 2000). In the developing diencephalon, PAX6 expression is restricted both antero-posteriorly and dorso-ventrally, and in homozygous Sey^{Neu} mice, there is a reduction in the discrete boundary between prosomeres 1 and 2, an expansion of the boundary region between prosomeres 2 and 3 (zona limitans), and a decrease in the zona inserta tissue (Grindley, Hargett, Hill, Ross, & Hogan,

1997). These mutants also provide evidence for the necessity of PAX6 in the formation of the posterior commissure, cumulatively demonstrating a role of PAX6 in diencephalon regionalization (Grindley et al., 1997).

Further loss-of-function studies have determined the necessity of PAX6 in cell proliferation, specification of dorsal and neurogenetic cell fates and appropriate migration of these neurons in the formation of the cortex (Gotz et al., 1998; Osumi, 2001; Schmahl et al., 1993; Warren, 1999). The role of PAX6 in neurogenesis is not restricted to development but persists through adult neurogenesis as well (Osumi, Shinohara, Numayama-Tsuruta, & Maekawa, 2008). This has been further characterized in the context of the dependence of progenitor cell orientation and division on PAX6, as well as the regulation of adhesive patterning via Pax6-mediated R-cadherin expression (Asami et al., 2011; A Stoykova, Gotz, Gruss, & Price, 1997). In addition to cellular migration, PAX6 has also been identified as a critical factor in cortical and cortico-thalamic axon guidance (Hevner, Miyashita-Lin, & Rubenstein, 2002; Jones, Lopez-Bendito, Gruss, Stoykova, & Molnar, 2002; Osumi, 2001). In superficial cortical layers which are generated during late corticogenesis, PAX6 is expressed at high levels. Further, overexpression of PAX6 at this stage of development reduces the number of cells in superficial cortical layers, and conditional deletion of PAX6 during corticogenesis results in decreased cell numbers as well as altered cell type specification in this region (Georgala, Manuel, & Price, 2011). Together these findings indicate that precise dosage effects of PAX6 are essential for normal development of the outer layers of the cortex (Georgala et al., 2011). In conditional knockouts of PAX6 during early and late corticogenesis (homozygotes of the $Pax6^{flox}$ allele under control of *Emx1-Cre* and *hGFAP-Cre* respectively), cortical layering abnormalities were determined to be a consequence of premature early cell cycle exit of progenitor cells during

formation of the lower cortical layers leading to an increase in these layers and oligodendrocyte differentiation as well as a decrease in the progenitor pool available for upper layer neuron specification and a reduction or absence of these layers (Tuoc et al., 2009). Further, knockouts under control of *Emx1-Cre* exhibited behavioral deficits in sensorimotor information integration, hippocampus-dependent short term memory processes and cortical-dependent long term memory processes (Tuoc et al., 2009). Cortex-specific knockout mice also demonstrate an enlargement of caudal cortical areas and a reduction in the rostral domain and, though supragranular cortical layers are decreased, the molecular identity of cortical neurons in the affected regions are not altered (Piñon, Tuoc, Ashery-Padan, Molnár, & Stoykova, 2008). Finally, cortex-specific knockout of PAX6 produces mice with decreased overall brain volume, olfactory bulb volume, cortical thickness and disorganization of interhemispheric fiber tracts as measured by magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) *in vivo* (Boretius et al., 2009).

As seen in the developing olfactory bulb, mice with homozygous loss-of-function mutations in PAX6 demonstrate abnormalities in catacholaminergic cell populations (epinephrine, norepinephrine and dopamine expressing cells) in discrete regions of the telencephalon, ventral thalamus and hypothalamus where PAX6 is expressed, as well as abnormal projections from catacholaminergic cells in the substantia nigra and ventral tegmental area despite the lack of PAX6 expression in these cells (Vitalis, Cases, Engelkamp, Verney, & Price, 2000). It follows that the severe neurodevelopmental phenotypes observed in PAX6 homozygous mutant mice are likely far-reaching consequences of early corticogenesis and proliferative processes which are directly dependent on precise spatiotemporal patterns and dosage of PAX6 expression. Further, defects in heterozygous mutants are exacerbated by decreased PAX6 function throughout life.

In the developing rhombencephalon Pax6 is highly expressed in the rhombomeric lip; the caudal lip becomes the precerebelar neuroepithelium and the rostral lip ultimately gives rise to granule cells in the normal developing cerebellum (Altman & Bayer, 1987; Zhang & Goldman, 1996). Experiments with heterozygous *Sey* mice carrying a lacZ reporter allele suggest that PAX6 is an upstream regulator of genes necessary for rhombomeric identity and segmentation of the developing hindbrain (Kayam et al., 2013). In homozygous *Sey* mice, three of five precerebellar nuclei do not form correctly, the cerebellar granule cell layer is absent, and some granule cells are found in ectopic locations in the inferior colliculus (Engelkamp, Rashbass, Seawright, & van Heyningen, 1999). These results demonstrate a necessary role of PAX6 in cerebellar neuronal migration and development, and the lack of *Unc5h3* (netrin receptor) expression indicates at least part of this phenotype is through PAX6-mediated regulation of *Unc5h3* (Engelkamp et al., 1999). Direct interactions of PAX6 with downstream targets such as *Unc5h*3 as well as assessment of global transcriptional networks provide avenues for investigating the molecular mechanisms underlying the necessity of PAX6 in the various developmental processes described above (Coutinho et al., 2011; Graw et al., 2005). This is further elucidated through studies of target gene networks specific to the different isoforms of PAX6 (Lakowski, Majumder, & Lauderdale, 2007).

Studies in *Drosophila* and chick further support the requirement of PAX6 (and PAX6 homologs) for normal neuroanatomical and functional development (Callaerts et al., 2001; Li, Yang, Jacobson, Pasko, & Sundin, 1994). These findings represent conserved mechanisms which extend to human development as demonstrated by human fetal immunohistochemical staining and *in vitro* testing. In the developing human brain, PAX6 is highly expressed in proliferating radial glia in dorsal and ventral proliferative zones, as well as deep cortical plate

neurons (Mo & Zecevic, 2008). In human radial glia *in vitro*, knockdown of PAX6 via siRNA decreases the number of differentiated neurons and implicates PAX6 in the generation of both pyramidal neurons and subpopulations of interneurons, the latter of which was previously not observed in rodent studies (Mo & Zecevic, 2008). Therefore, while studies in model organisms are useful for characterizing conserved aspects of PAX6-dependent processes in the brain, the full range of consequences of PAX6 mutations must be directly observed in humans.

1.3 Neuroanatomical and neurocognitive phenotypes in aniridia

1.3.1 Structural abnormalities and functional consequences

The primary feature of aniridia is a hypoplastic or absent iris, and persons with aniridia exhibit a number of other ocular as well as pancreatic symptoms which characterize the disorder (Hingorani, Williamson, Moore, & van Heyningen, 2009; Jordan et al., 1992; Nishi et al., 2005; Peter et al., 2013). However, phenotypes associated with the brain are currently a topic of great interest and are becoming a central component in understanding the composite symptoms of aniridia. Structural MRI studies have identified abnormalities in specific brain structures, white matter tracts, and whole neuroanatomical regions in persons with PAX6-mediated aniridia. Pronounced abnormalities in interhemispheric white matter tracts have been observed, including hypoplasia or absence of the anterior commissure, reduction of the posterior commissure, agenesis of the corpus callosum, and atrophy of the optic chiasm (Abouzeid et al., 2009; Bamiou et al., 2004, 2007; Mitchell et al., 2003; Sisodiya et al., 2001). The most consistent of these structural alterations in white matter across studies is seen in the anterior commissure, and the resulting impairment of interhemispheric transfer has been causally implicated in cognitive processing deficits in working memory and auditory processing (Bamiou et al., 2007; Thompson et al., 2004). Another finding in this population which has been replicated is an absence or

hypoplasia of the pineal gland; an endocrine gland involved in the regulation of circadian rhythm (Abouzeid et al., 2009; Hanish, Butman, Thomas, Yao, & Han, 2015; Mitchell et al., 2003). This finding, combined with impaired ability to regulate light due to lack of iris, is the likely cause of disordered sleep reported by persons with aniridia (Hanish et al., 2015; Ross, 1998; Wee & Van Gelder, 2004). Unilateral polymicrogyria and reduced olfactory function have also been observed in this population (Mitchell et al., 2003; Sisodiya et al., 2001). In a quantitative voxel-based morphometry (VBM) study of PAX6-mediated aniridia, bilateral increases in cerebellar grey matter volume and ventral occipital cortex, white matter reduction in the corpus callosum, and abnormal sulcus orientations in the occipital lobe were reported (Free et al., 2003). This study also detected differences between a group of persons with C-terminal extension mutations and those with predicted haploinsufficiency in occipital and cerebellar grey matter (Free et al., 2003). These differences, along with the variation seen in individual affected structures, suggest that some neuroanatomical brain abnormalities in aniridia may be consistent in the population, while others may be associated with specific types of mutations. The investigation of this question demands closer inspection of human mutations at the molecular level, as well as the use of model organisms to dissociate conserved PAX6-dependent neuroanatomical abnormalities from mutation-specific or modifier effects that are unique to humans.

1.3.2 Neuroimaging techniques towards understanding neural phenotypes

While a number of studies have examined neuroanatomical abnormalities in aniridia, the functional link between these changes and cognitive and sensory processing deficits remains largely correlative. Our work presented the first functional assessment of neural activity in this population using functional MRI (fMRI). We showed that in resting state functional networks, increases in connectivity representing recruitment of non-traditional neuroanatomical areas were

present in persons with aniridia relative to subject-matched controls in the executive control, primary visual and default mode networks (Pierce et al., 2014). These findings are the first to directly examine brain activity in aniridia, and likely capture the functional consequences of gross neuroanatomical findings and neuronal circuit disruptions which are not detectable by structural MRI. Prior to the current work, functional neuroimaging measures in the context of cognitive or sensory processing applied to this population remained entirely unexplored. Electroencephalography (EEG) provides a direct measure of neural activity with incredible temporal sensitivity, and can be implemented in conjunction with stimuli which selectively target cognitive or sensory processes. Together with molecular and neuroanatomical techniques, functional neuroimaging measures such as fMRI and EEG provide a perspective on neurocognitive processes that can considerably advance our understanding of extraocular phenotypes in PAX6-mediated aniridia.

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CHAPTER 2:

ASSESSMENT OF PAX6 ALLELES IN 66 FAMILIES WITH ANIRIDIA[1]

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Abstract

We report on *PAX6* alleles associated with a clinical diagnosis of classical aniridia in 81 affected individuals representing 66 families. Allelic variants expected to affect PAX6 function were identified in 61 families (76 individuals). Ten cases of sporadic aniridia (10 families) had complete (8 cases) or partial (2 cases) deletion of the *PAX6* gene. Sequence changes that introduced a premature termination codon into the open reading frame of *PAX6* occurred in 47 families (62 individuals). Three individuals with sporadic aniridia (3 families) had sequence changes (1 deletion, 2 run-on mutations) expected to result in a C-terminal extension. An intronic deletion of unknown functional significance was detected in one case of sporadic aniridia (one family), but not in unaffected relatives. Within these 61 families, single nucleotide substitutions accounted for 30/61 (49%), indels for 23/61 (38%), and complete deletion of the *PAX6* locus for 8/61 (13%). In five cases of sporadic aniridia (five families), no disease-causing mutation in the coding region was detected. In total, 23 unique variants were identified that have not been reported in the Leiden Open Variation Database (LOVD) database. Within the group assessed, 92% had sequence changes expected to reduce PAX6 function, confirming the primacy of PAX6 haploinsufficiency as causal for aniridia.

2.1 Introduction

Aniridia is a congenital, progressive disorder for which the majority of cases are caused by heterozygous loss-of-function mutations of the *PAX6* gene (I. M. Hanson et al., 1993; Parekh et al., 2015). Aniridia occurs in approximately 1/64,000 to 1/96,000 live births and is primarily characterized by iris hypoplasia which is clinically detectable at birth (Parekh et al., 2015; Pozdeyeva, Pashtayev, Lukin, & Batkov, 2005). Associated foveal hypoplasia, indicated by early infancy nystagmus, causes reduced visual acuity. The progressive nature of the disease frequently leads to multiple ocular abnormalities such as keratopathy, corneal vascularization and opacification, glaucoma, anterior chamber fibrosis, and cataracts (Grant & Walton, 1974; McCulley, Mayer, Dahr, Simpson, & Holland, 2005; Nishida, Kinoshita, Ohashi, Kuwayama, & Yamamoto, 1995; Parekh et al., 2015; Tsai et al., 2005). Along with distinct ocular characteristics, the condition is associated with a number of other irregularities spanning sensory, neural, cognitive (Thompson et al., 2004) and pancreatic phenotypes (Ashery-Padan et al., 2004; Nishi et al., 2005). These include auditory processing deficits (Bamiou et al., 2004, 2007), deficient pituitary function (Shimo et al., 2014) and olfactory dysfunction (Sisodiya et al., 2001). Previous studies have identified a correlation between *PAX6*-mediated aniridia and a number of other disorders, including diabetes (Peter et al., 2013) and autism spectrum disorders (Davis et al., 2008). In addition, structural abnormalities in major fiber tracts and subcortical structures in the brain including the corpus callosum, anterior and posterior commissures, pineal gland and probst bundles have been observed (Mitchell et al., 2003; Sisodiya et al., 2001). A functional understanding of the *PAX6* gene in context of the manifestation of aniridia-related clinical traits continues to be the focus of much research.

Functional mutations that occur only in the *PAX6* gene or associated regulatory regions give rise to aniridia, which can be sporadic or familial. Wilms tumor, aniridia, genitourinary anomalies and mental retardation syndrome (WAGR) is associated with large heterozygous genomic deletions at the 11p13 chromosome that include deletion of the *PAX6* locus and the *WT1* locus (Fischbach, Trout, Lewis, Luis, & Sika, 2005; Riccardi, Sujansky, Smith, & Francke, 1978). In addition to clinical symptomologies associated with aniridia, patients with WAGR syndrome present with Wilms tumor and, depending on the size of the causal chromosomal deletion, often exhibit cognitive delays and disabilities in addition to developmental genitourinary defects (Fischbach et al., 2005). Aniridia is not comorbid with WAGR syndrome; however, it can be further classified at the cellular and molecular level by the specific mutation affecting the *PAX6* gene.

The *PAX6* gene encodes a transcription factor critical for normal ocular and neural development. The gene is highly conserved and is expressed in the developing eye, brain, spinal cord and pancreas (Kim & Lauderdale, 2006). PAX6 is required for various aspects of anatomical and functional development. The mechanistic role of PAX6 in eye development has been investigated and its involvement has been demonstrated in initial lens development (Ashery-Padan & Gruss, 2001), cell differentiation (Ericson et al., 1997; Higgins et al., 1994; Kohwi, Osumi, Rubenstein, & Alvarez-Buylla, 2005), and cell proliferation and adhesion/migration (Mastick, Davis, Andrew, & Easter, 1997). PAX6 has also been identified as having critical maintenance functions in corneal homeostasis (Collinson, Chanas, Hill, & West, 2004; Koroma, Yang, & Sundin, 1997). In addition, in the central nervous system, PAX6 is thought to be involved in brain patterning and regionalization and the formation of neural circuits, particularly in the forebrain (Manuel & Price, 2005; Mastick et al., 1997; Osumi, 2001;

Simpson & Price, 2002). In these ways, PAX6 is an integral upstream regulator involved in numerous developmental gene networks. It follows that the broad developmental and progressive phenotypic outcomes caused by *PAX6* mutations are a consequence of the extensive roles of the gene and its products.

The PAX6 protein is composed of four functional domains: a paired domain and a homeodomain (involved in DNA binding) joined by a linker domain, and a proline-serine-threonine (PST)-rich transactivation domain (Tang, Singh, & Saunders, 1998). Should products of mutant alleles undergo translation, disruptions in the coding sequence of different functional domains will alter the efficacy of PAX6 as a transcriptional regulator, which has been shown in molecular and phenotypic studies of missense and run-on *PAX6* mutations in aniridia and other related disorders (Aggarwal, Jinda, Limwongse, Atchaneeyasakul, & Phadke, 2011; Azuma et al., 1999; Azuma, Nishina, Yanagisawa, Okuyama, & Yamada, 1996; Chao, Mishra, Strong, & Saunders, 2003; I. Hanson et al., 1999; Maekawa et al., 2009; Singh, Chao, Mishra, Davies, & Saunders, 2001; Tang, Chao, & Saunders, 1997). However, the majority of *PAX6*-mediated aniridia cases are caused by mutations that give rise to premature termination codons (PTCs) leading to haploinsufficient levels of wild-type PAX6 protein.

Prior to the current study, the Human *PAX6* Allelic Variant Database (LOVD database) (http://lsdb.hgu.mrc.ac.uk/home.php?select_db=PAX6) has identified 361 unique variants of *PAX6* (833 total reported). This study sought to further examine and classify mutations of *PAX6* in patients diagnosed with classical aniridia, as well as examine our data in the context of previously identified mutations of the gene. This line of investigation provides insights towards identifying locations on the *PAX6* gene that are more susceptible to mutations and seeks to

explore individual-specific mutation characteristics which could benefit from new therapeutics within the aniridia population.

2.2 Materials and methods

2.2.1 Participants

DNA samples were collected from 81 individuals with a clinical diagnosis of aniridia and 77 unaffected genetic relatives from 66 families (Table S1, Supporting information). Participants were recruited through the Aniridia Foundation International Conferences in 2007, 2009, and 2011 through affiliation and direct physician referral for participation in the study. The study protocol was in keeping with the tenets of the Declaration of Helsinki and was reviewed, approved, and overseen by the Institutional Review Boards at the University of Tennessee Health Science Center and the University of Georgia. All samples were collected after written informed consent had been obtained from each participant.

2.2.2 DNA Preparation

Genomic DNA from individuals with aniridia and their relatives was prepared from peripheral venous whole blood. Briefly, 10mL volume blood samples were collected from each adult participant, and 2mL blood samples were collected from each infant. Buffy coat preparation was performed on each sample and genomic DNA (gDNA) prepared by use of a Gentra Puregene Blood Kit (Qiagen). Buccal cell lysates were prepared from saliva or swab samples (Oragene-DNA OG-575, DNA Genotek, Inc, Kanata, Ontario, Canada; buccal swabs and QuickExtract DNA extraction solution, Epicentre, Madison, WI , U.S.A.). All gDNA samples were stored in 10 mM Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA) buffer at -20°C.

2.2.3 Molecular Analysis

The *PAX6* gene (11p13) [OMIM: 607108] is composed of sixteen exons (Table S2) (Kim & Lauderdale, 2006), of which 11 (4 to 13 and 5a) contribute to the protein-coding regions of the mRNA transcripts. The other exons (0, 1, 2, 3, and *alpha*) contribute to the non-coding regions of the mRNA transcripts. For this study, *PAX6* exons 1-13 and 5a were individually amplified in a polymerase chain reaction (PCR) with primers located in the introns as previously described (Glaser, Walton, & Maas, 1992; Love, Axton, Churchill, van Heyningen, & Hanson, 1998). Mutation screening was performed on the PCR products by direct bidirectional sequencing using either the same PCR primers or more internal primers (Glaser et al., 1992; Love et al., 1998). Bidirectional sequence was analyzed and compared to *PAX6* reference sequences. Reference sequences for *PAX6* cDNA and protein are contained in GenBank entry NM_000280 (http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). The February 2009 human reference sequence GRCh37 produced by the Genome Reference Consortium (http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/) was used as the *PAX6* genomic reference after first validating against genomic sequences obtained from cosmids FAT5 and A1280 (Genbank accession numbers Z95332 and Z83307, respectively), which together encompass all *PAX6* exons. Table S2 lists intron-exon boundary sequences for human *PAX6* and all exon sizes. Potential mutations were identified as differences relative to the appropriate reference sequence. Results were confirmed by repeat PCR, which in most cases was performed using an independent, second gDNA sample prepared from either blood or buccal cells. Changes from the reference sequence were identified, classified and are documented in Table 1 using the nomenclature system recommended by the Human Genome Variation Society (den Dunnen &

Antonarakis, 2000), with the first base of the ATG initiation codon in the *PAX6* cDNA reference sequence NM_000280 denoted as nucleotide 1.

Deletion/duplication testing was performed either by array-comparative genomic hybridization (CGH) analysis (Xu et al., 2008) (GeneDx or Emory Genetics Laboratory), fluorescence in situ hybridization (Crolla et al., 1997), or physical mapping (Lauderdale, Wilensky, Oliver, Walton, & Glaser, 2000).

Individuals for which no *PAX6* pathogenic mutations were detected were retested using fresh samples by GeneDx or the Denver Genetic Laboratories (University of Colorado Anschutz Medical Campus).

2.3 Results

A total of 158 individuals (81 affected, 77 unaffected genetic relatives) from 66 families were evaluated in this study (Table S1). All affected individuals had a clinical diagnosis of aniridia (Figure 1, data not shown). In the context of this study, "family" was used to group genetically related individuals, and includes instances of sporadic aniridia (a single individual within the family) or familial aniridia (multiple individuals). Of the 81 affected individuals, 53 were female and 28 were male. Fifty-eight cases were sporadic (44 female, 14 male), with no affected relatives at the time of birth. Of these, five females subsequently had children (two female, four male) with aniridia. Fifteen additional cases (six female, nine male) were familial from four separate families. The parents for two cases (one female, two male) were unknown, and so it is not possible to determine if these two individuals were sporadic or not.

The *PAX6* gene was characterized for all individuals. Sequence changes relative to the appropriate *PAX6* reference sequence (see Materials and methods) were identified and classified (Table 1, Table S1). Potential aniridia-causing changes were identified as differences relative to

the reference sequence and sequences from unaffected genetically related family members. Sequence variants were identified in individuals with aniridia in 64 of the 66 families (79 out of 81 individuals). One of these variants (c.766-12C>T) was detected in both affected and unaffected individuals in three families (three individuals with sporadic aniridia) and is therefore likely to be a benign sequence change (Table 1C), as has been previously suggested (Dharmaraj et al., 2003; Kokotas & Petersen, 2010; Villarroel et al., 2008). No sequence changes were detected in unaffected relatives in the remaining 63 families. Thus aniridia-specific alterations in the *PAX6* gene were identified in 61 families (76 individuals).

Sequence alterations predicted to cause loss-of-function of one copy of the *PAX6* gene were identified in 57 families (72 individuals). Whole gene deletions were detected in eight cases of sporadic aniridia (eight families); seven of these deletions also included the *WT1* gene. A partial deletion of *PAX6* was detected in two cases of sporadic aniridia (two families). In one case, the region extending from the P1 promoter to exon 4 was deleted, and in the other, a region encompassing exons 6 and 7 was deleted. Nonsense mutations were identified in 18 families (24 individuals), and frameshifting deletions or insertions were identified in 16 families (23 individuals). Changes expected to disrupt normal splicing were identified in 13 families (15 individuals). Of these, nine were changes to the dinucleotides in the 5' and 3' splice sites. Also included were a C to T transition at the -3 position in the 5' flanking sequence of exon 10 (c.766-3C>T, family 173) and a T to G transversion at the +6 position in the 3' flanking sequence of exon 11 (c.1032+6T>G, family 130). These two variants were included because changes in these positions are known to affect splicing in other human genes (Ward & Cooper, 2010) and there is a high degree of conservation in the sequences immediately flanking *PAX6* exons between humans, dogs, mice, chickens, *Xenopus tropicalis*, and zebrafish (data not shown). In

families 117, 143, 163, and 169, where deletions included both exonic and intronic sequences, the deletion was scored as 'frameshifting' if the deletion originated within the exon and included the 3' flanking sequence (families 143 and 163) or 'splice junction' if the deletion originated in the intron and included the 5' end of the subsequent exon (families 117 and 169).

Mutations predicted to result in a PAX6 protein with a C-terminal extension were identified in three cases of sporadic aniridia. Two cases had an A to T transversion (c.1268A>T) that converts the stop codon (TAA) to a leucine codon (TTA). This mutation is predicted to result in the addition of 14 residues to the C-terminal portion of the PAX6 protein. One case had a single nucleotide deletion (c.1256delC) that is predicted to result in a frameshift at amino acid 419 and the addition of 105 residues to the C-terminal portion of the PAX6 protein.

One case of sporadic aniridia (family 128) had a heterozygous deletion of the 18^{th} - 20^{th} nucleotides in the intronic sequence just 3' to exon 5 (c.141+18_+20delGCC). This deletion was not present in unaffected family members and is, therefore, likely to be causal for aniridia in this individual. Although the molecular effect of this change is not known, its position within the intron may affect splicing or alter a regulatory element.

The exon-by-exon distribution of single nucleotide substitutions and small indels (≤150 bp) associated with aniridia that were found in 51 families is shown in Figure 2. Mutations were detected in all exons except for 4, 5a and 9. Twenty-one variants (41%) occurred within the paired domain, 11 variants (22%) occurred within the homeodomain, 8 (16%) within the linker domain, and 11 variants (22%) occurred within the C-terminal transactivation domain (Figure 3). In total, 23 unique variants, not including gross deletions, were identified which have not been previously reported in the LOVD database (Table 1: variants in bold).

2.4 Discussion

In the group of individuals with a diagnosis of classical aniridia analyzed here, aniridia-associated alterations in the *PAX6* gene were identified in 61 out of 66 families (76 out of 81 individuals). These changes included complete (8 instances) or partial deletion (2 instances) of the *PAX6* gene, introduction of a PTC into the open reading frame of *PAX6* (47 instances), and likely addition of residues to the C-terminal end of the PAX6 protein (3 instances). These changes are all predicted to reduce PAX6 function. In addition, an intronic deletion of unknown functional significance was detected in one case of sporadic aniridia (one family), but not in unaffected relatives. Overall, the distribution of the variants identified in the current study demonstrates similar characteristics when compared with the LOVD database (Figure 2a, b), except that the comparative frequency of frame-shifting insertions or deletions is higher in this study. These results confirm the primacy of *PAX6* haploinsufficiency as causal for aniridia.

No pathological sequence changes were observed in 5 families with aniridia. At this time, these individuals appear clinically indistinguishable from others with aniridia who have known *PAX6* mutation (Figure 1, data not shown). Three of these individuals are children and it is possible that clinical differences will emerge as they get older. These persons could harbor mutations in other genes involved in anterior eye development, such as *FOXC1* or *PITX3*, among others, or they may have mutations in *PAX6* regulatory elements. We are currently exploring these possibilities.

The major mutational mechanisms underlying genetic variation are single nucleotide substitutions (Sachidanandam et al., 2001; The International HapMap Consortium, 2005; "Nature," 2003), small DNA insertions and deletions (indels) ranging from 1 to 10,000 bp in length (Bhangale, Rieder, Livingston, & Nickerson, 2005; Mills et al., 2006; Mullaney, Mills,

Pittard, & Devine, 2010; Weber et al., 2002), and large scale (>10 kb) genome rearrangements (Conrad, Andrews, Carter, Hurles, & Pritchard, 2006; Hinds, Kloek, Jen, Chen, & Frazer, 2006; Iafrate et al., 2004; Kidd et al., 2008; McCarroll et al., 2005; Sebat et al., 2004; Tuzun et al., 2005). For the 61 families with aniridia-specific alterations in the *PAX6* gene, single nucleotide substitutions accounted for 30/61 (49%), indels accounted for 23/61 (38%), and gross deletion of the *PAX6* locus accounted for 8/61 (13%) of instances. These results demonstrate that single nucleotide substitutions and indels in *PAX6* are a significant source of aniridia-causal mutations. Of the single nucleotide substitutions, 13/30 (43%) occurred at CpG dinucleotides, a structure known for its high mutability in the human genome (Cooper & Youssoufian, 1988; Nachman & Crowell, 2000) and to be associated with significant mutation hotspots in *PAX6* (Prosser & van Heyningen, 1998; Tzoulaki, White, & Hanson, 2005). Consistent with previous reports (Prosser & van Heyningen, 1998; Tzoulaki et al., 2005), nonsense mutations in exons 8, 10 and 11 occurred at CpG locations that created arginine CGA codons Arg203 (c.607C>T, 2 instances), Arg261 (c.781C>T, 4 instances) and Arg317 (c.949C>T, 3 instances), respectively. In addition, a CpG located at the 3' exon/intron junction of exon 6 was mutated in four families (c.357+1G>A, 3 instances; c.357+1G>T, one instance). The frequency of indels is notable as it is about twofold more than has been reported for the genome at large (Mullaney et al., 2010). Because indels result from nonhomologous end joining as a result of DNA break repair, the high frequency of indels in *PAX6* suggests that the 11p13 region of human chromosome 11 may be unstable relative to other regions of the genome. Alternatively, the frequency of indels in the human genome may currently be underreported due to technical issues with detecting these types of changes throughout the genome (Mills et al., 2011). Indels have been reported to occur

preferentially in the male germ line (Messer & Arndt, 2007), and may be useful in tracking inheritance.

Analysis of the different types of mutations found in this study, in conjunction with those previously reported in the LOVD database can help to differentiate possible functional implications of the nature of mutations, in the context of phenotype analysis as well as potential genetic treatments. This requires consideration of individual mutations in the context of molecular and cellular mechanisms that act on mutant gene products. Mutations that lead to a PTC give rise to mRNA transcripts, which are putative targets of nonsense-mediated mRNA decay (NMD). NMD is an RNA-surveillance pathway which recognizes and degrades transcripts with PTCs upstream of the last exon-junction complex (EJC) during the primary round of translation (Chang, Imam, & Wilkinson, 2007). This mechanism serves to prevent the translation of truncated proteins with possible dominant negative effects and is expected to target all transcripts with a PTC upstream of the last EJC regardless of the nature of the causal mutation. In patients with mutations fitting these criteria, degradation of transcripts from the mutant *PAX6* allele should result in *PAX6* haploinsufficiency. However, *PAX6* transcripts harboring mutations upstream of the coding region of *PAX6*, those causal for PTCs near or downstream of the last EJC, or missense mutations would likely evade NMD and could produce dysregulated or truncated dominant negative PAX6 protein products from the mutant allele. Three cases in the current study previously mentioned as causal for C-terminus extensions of the open reading frame would fall into this category, leaving the vast majority of cases subject to this RNA surveillance pathway and ultimately leading to *PAX6* haploinsufficiency. Dominant negative PAX6 proteins could interfere with proteins produced from the wild-type allele, especially if the DNA binding domain structures remain intact. Future studies will seek to

compare phenotypic differences at the levels of both cellular/molecular differences and clinical presentation between patients with and without mutations identified as putative NMD targets.

In addition to contributing to our understanding of *PAX6* allelic differences in the context and implications of haploinsufficiency, knowledge about the different disease-causing mutations is necessary for the development of potential therapeutic approaches to treat individuals with aniridia. Approaches that could be used include: (i) those that are cell-based coupled with genome editing or manipulation, (ii) those designed to increase *PAX6* expression from the normal allele, through the use of microRNAs for example, and (iii) those targeted towards generating functional PAX6 protein from the mutant transcripts.

Recently, a novel therapeutic known as ataluren (formerly PTC124) has been identified as a treatment for genetically-mediated diseases which are caused by mutations leading to a PTC (Welch et al., 2007). With successful clinical applications for other diseases (Bushby et al., 2014; Kerem et al., 2014; Sermet-Gaudelus et al., 2010; Wilschanski et al., 2011) as well as evidence of the efficacy of ataluren in mouse models of aniridia (Gregory-Evans et al., 2014), data from the current study could serve to identify potential candidates for this treatment based on specific genetic mutations of *PAX6*. Although ataluren has been demonstrated to effectively promote read-through of all PTCs, it shows the most significant increase in read-through when the PTC consists of the UGA nucleotide sequence (followed in significance by UAG and then UAA) (Welch et al., 2007). The efficacy of ataluren on promoting read-through of PTCs (via START drug formulation) specifically in the *PAX6* gene has recently been shown in a mouse model of aniridia possessing a nonsense mutation which induces a UGA in-frame premature stop codon (Gregory-Evans et al., 2014). Further, administration of this drug to mice with a mutant *PAX6* allele provides demonstrable evidence for partial rescue of ocular phenotypes associated

with aniridia, as a product of both systemic and topical postnatal application (Gregory-Evans et al., 2014). These results are promising for the treatment of human aniridia with START therapy in patients with nonsense mutations, especially those consisting of an UGA in-frame premature stop codon. The current study has identified 14 families (20 individuals) with a UGA in-frame premature stop codon who may be good candidates for this therapy, followed by 3 families (3 individuals) with an UAG in-frame premature stop and 1 family (1 individual) with an UAA in-frame premature stop.

Results of this study have identified new variants in the human *PAX6* gene causal for aniridia and, in conjunction with previously identified mutations, serve to further the understanding of *PAX6* mutations in this disorder as well as inform future studies of novel pharmacological treatments which may be beneficial for the treatment of aniridia.

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Table 1A: PAX6 variants expected to cause pathology.

FID	*LOCATION*	*VARIANT*	*TYPE OF VARIANT*	*PREDICTED EFFECT*
157	Exon 5	c.28C>T	transition	nonsense
118	Exon 5	c.57delG	indel	frameshift deletion
108	Exon 5	**c.63-70delGCCGGACT**	indel	frameshift deletion
133	Exon 5	c.112delC	indel	frameshift deletion
101	Exon 5	**c.112-116delCGGCC**	indel	frameshift deletion
135	Exon 5	**c.121_122insGCGG**	indel	frameshift insertion
138	Intron 5	c.141+1G>T	transversio	splice junction disruption
152	Intron 5	**c.141+2_+30delTGATCCTCCCGGCGCCG CCCCACTCGCCG**	indel	splice junction disruption
128	Intron 5	**c.141+18_+20delGCC**	indel	Unknown effect
150	Exon 6	**c.179-185delATTACGAinsCTGAT**	indel	frameshift

				deletion/insertion
112	Exon 6	c.199A>T	transversio	nonsense
147	Exon 6	**c.204delC**	indel	frameshift deletion
155	Exon 6	**c.332insG**	indel	frameshift insertion
1003	Exon 6	**c.343delG**	indel	frameshift deletion
163	Exon 6 & Intron 6	**c.352-357+2delCCAAGCGT**	indel	frameshift deletion
100	Intron 6	c.357+1G>A	transition	splice junction disruption
129	Intron 6	c.357+1G>A	transition	splice junction disruption
230	Intron 6	c.357+1G>A	transition	splice junction disruption
105	Intron 6	c.357+1G>T	transversio	splice junction disruption
169	Intron 6 & Exon 7	**c.358-3_361delCAGGTGT**	indel	splice junction disruption

168	Exon 7	c.365C>A	transversio	nonsense
141	Exon 7	**c.401delA**	indel	frameshift deletion
104	Exon 7	**c.454C>T**	transition	nonsense
126	Exon 7	c.467G>A	transition	nonsense
127	Exon 7	**c.480delT**	indel	frameshift deletion
114	Exon 7	c.482delG	indel	frameshift deletion
117	Intron 7 & Exon 8	c.524-101_534del112bp	indel	splice junction disruption
102	Exon 8	c.607C>T	transition	nonsense
160	Exon 8	c.607C>T	transition	nonsense
189	Exon 8	**c.631C>T**	transition	nonsense
173	Intron 9	c.766-3C>G	transversio	splice junction disruption
124	Exon 10	**c.771delG**	indel	frameshift deletion
144	Exon 10	c.781C>T	transition	nonsense
164	Exon 10	c.781C>T	transition	nonsense
170	Exon 10	c.781C>T	transition	nonsense

232	Exon 10	c.781C>T	transition	nonsense
110	Exon 10	c.794G>A	transition	nonsense
227	Exon 10	c.795G>A	transition	nonsense
191	Exon 10	c.799A>T	transversio	nonsense
103	Exon 10	**c.802_806delGAAGA**	indel	frameshift deletion
120	Exon 11	c.949C>T	transition	nonsense
153	Exon 11	c.949C>T	transition	nonsense
1001	Exon 11	c.949C>T	transition	nonsense
130	Intron 11	**c.1032+6T>G**	transversio	splice junction disruption
122	Intron 11	c.1033-2A>G	transition	splice junction disruption
143	Exon 12 & Intron 12	**c.1174_+6delACTTCAACAGGTGAGC**	indel	frameshift deletion
119	Intron 12	**c.1183+1G>A**	transition	splice junction disruption
1004	Intron 12	**c.1183+1G>A**	transition	splice junction disruption
185	Exon 13	**c.1256delC**	indel	frameshift deletion; C-

				terminal extension
115	Exon 13	c.1268A>T	transversio	run-on, C-terminal extension
140	Exon 13	c.1268A>T	transversio	run-on, C-terminal extension

Table 1B: Partial and gross deletions of PAX6.

FID	*LOCATION*	*VARIANT*	*TYPE OF VARIANT*	*PREDICTED EFFECT*
228		**Deletion P1 promoter to exon 4**	indel	
1002		**Deletion exons 6 and 7**	indel	
161		**WT1-PAX6; 3' extent unknown**	gross deletion	loss-of-function
174		**WT1-PAX6; 3' extent unknown**	gross deletion	loss-of-function
180		**WT1-PAX6; 3' extent unknown**	gross deletion	loss-of-function

182		**WT1-PAX6; 3' extent unknown**	gross deletion	loss-of-function
214		**WT1-PAX6; 3' extent unknown**	gross deletion	loss-of-function
219		**WT1-PAX6; 3' extent unknown**	gross deletion	loss-of-function
225		**WT1-PAX6; 3' extent unknown**	gross deletion	loss-of-function
501		**Deletion of PAX6**	gross deletion	loss-of-function

Table 1C: Families with no pathological mutations of PAX6 detected.

FID	*LOCATION*	*VARIANT*	*TYPE OF VARIANT*	*PREDICTED EFFECT*
121		No changes detected		N/A
162	Intron 9	c.766-12C>T	transition	Variant of unknown significance
166	Intron 9	c.766-12C>T	transition	Variant of unknown significance
190	Intron 9	c.766-12C>T	transition	Variant of unknown significance
231		No changes detected		N/A

Table 2.1 Identified variants in this study. (A) 51 sequence variants (from 51 families) sorted by genomic location and are described by type of variant, intronic/exonic location, and the predicted effect on gene products. (B) Complete and partial deletions of PAX6 from 10 families. (C) Five families with no pathological change in PAX6 detected. Variants in bold represent those not previously identified in the LOVD database.

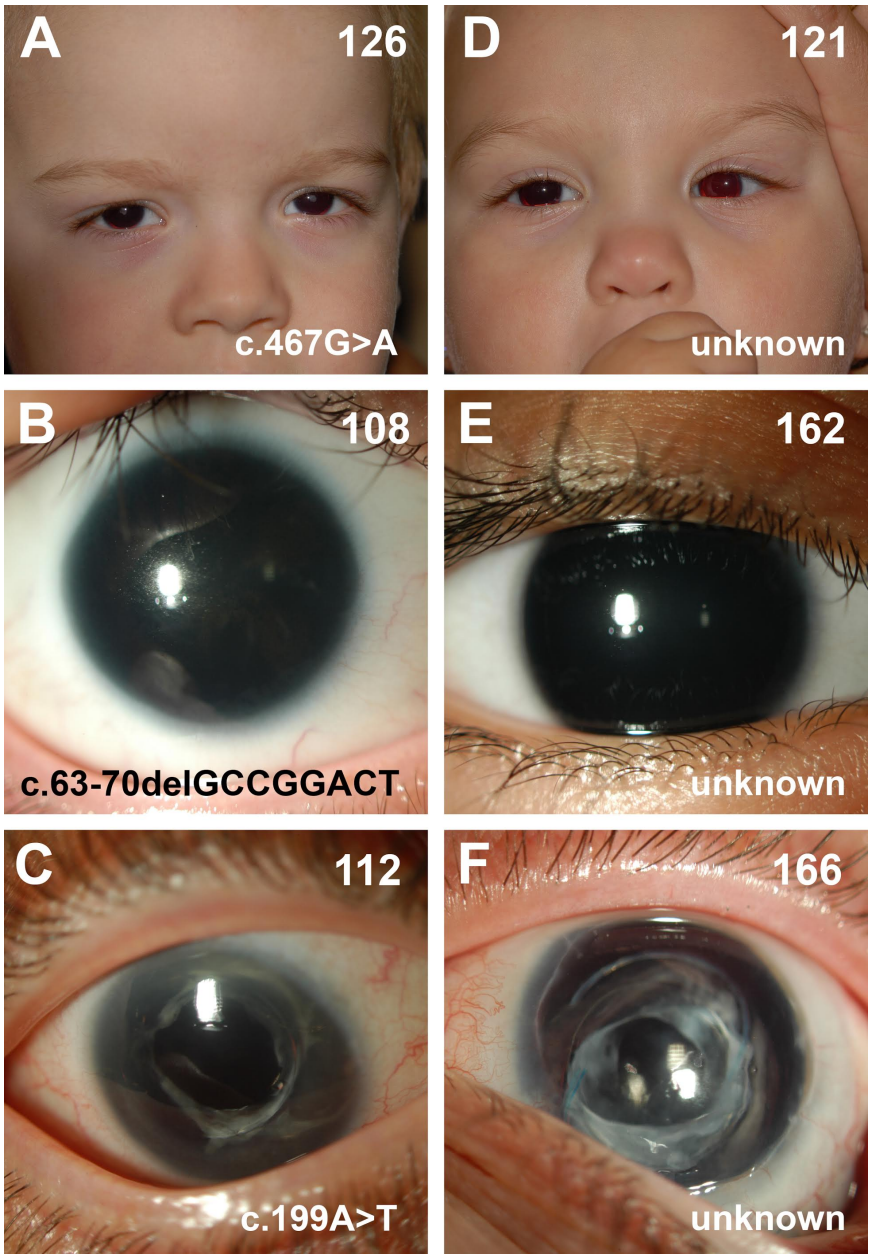

Figure 2.1. Ocular phenotypes associated with individuals harboring *PAX6* coding mutations (A-C) and those for whom no coding mutations were detected (D-F). Images from individuals with known *PAX6* mutations were chosen to facilitate comparison with panels D-F and do not capture the full range of ocular phenotypes observed in individuals with identified mutations of the *PAX6* gene. Family ID, upper right; causal sequence variant, lower right.

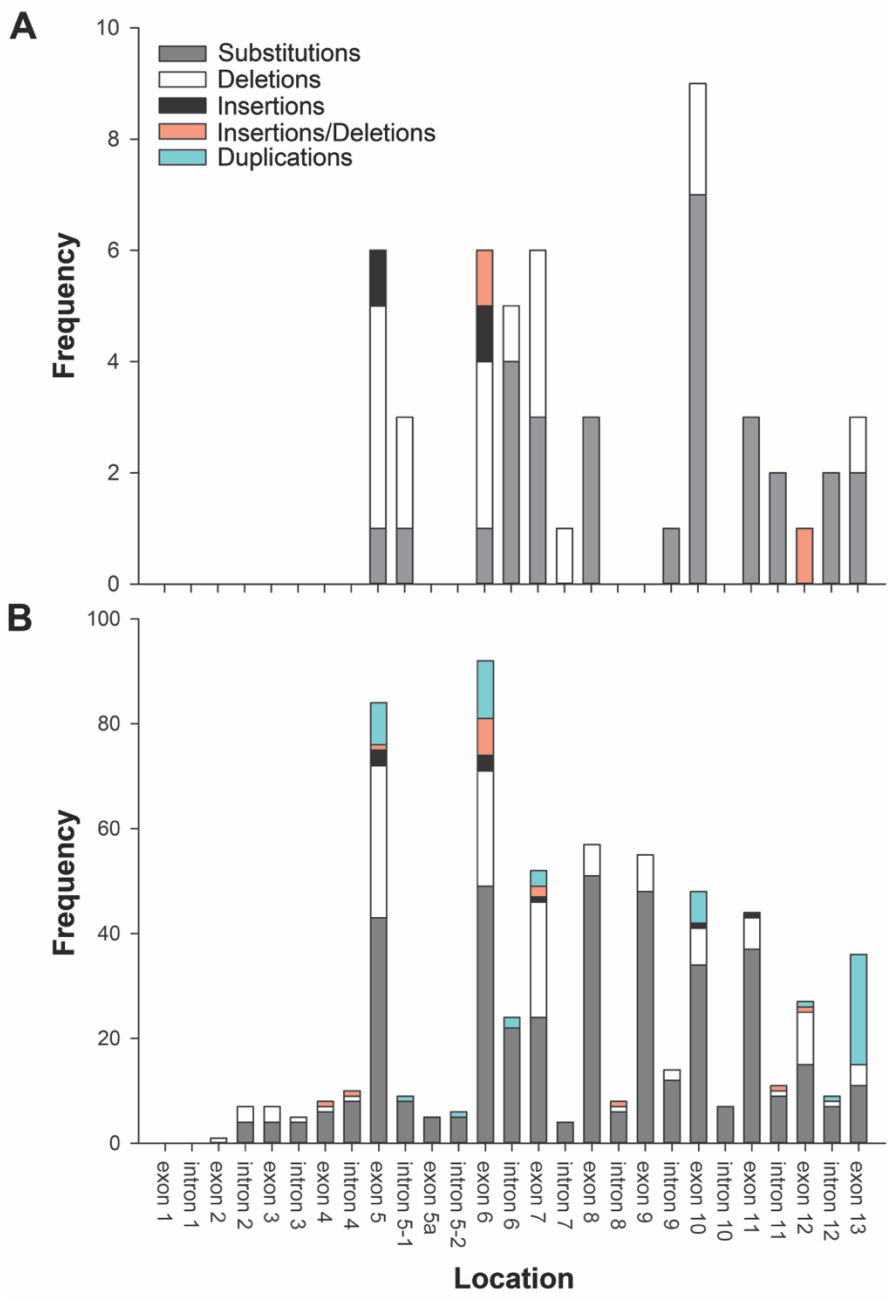

Figure 2.2. Distribution of *PAX6* variants. (A) Frequency of pathological variants identified in the current study located in each exon and intron of the *PAX6* gene (N=51). (B) Frequency of variants complied from the PAX6 Allelic Variant Database as of June 2, 2015 (N=623). Variants classified based on nature of sequence alteration.

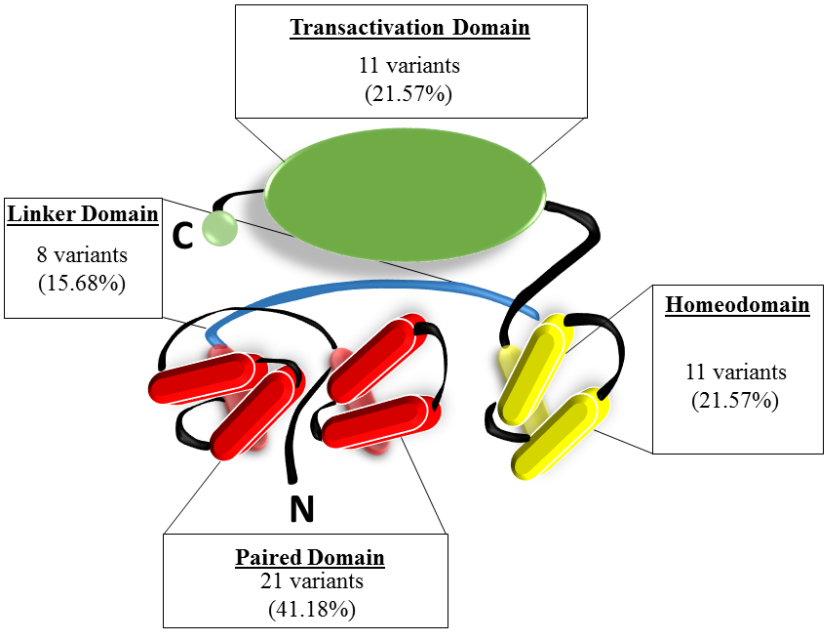

Figure 2.3. Summary of allelic variants predicted to affect each protein domain. Illustration of the PAX6 protein with summary statistics of the variants in the current study predicted to affect protein structure in (A) the paired domain, (B) the linker domain (C) the homeodomain, and (D) the transactivation (PST) domain.

FID	*Person ID*	*Sex*	*Inheritance*	*Variant*	*Varient Sequence*
100	100A151/s-100	F	Sporadic	c.357+1G>A	CATACCAAGC/**a**taagttcattg
101	101A111/s-101	F	Sporadic	c.112-116delCGGCC	GCGGGGCC(CGGCC)GTGCGACATTTCCCGAATTCTGCAG/
102	102A127/s-102	F	Sporadic	c.607C>T	CAA ATG **T**GA CTT CAG
103	103A025/s-103	M	Sporadic	c.802_806delGAAGA	CGA AGG GCC AAA TGG AGA AGA (GAA GA)A AAA CTG
104	104A098/s-104	M	Sporadic	c.454C>T	AAC GGG **T**AG ACC GGA
105	105A046/u-105	F	Parents Unknown	c.357+1G>T	CATACCAAGC/**t**taagttcattg
108	108A128/f-108	M	Familial	c.63-70delGCCGGACT	GGG CGG CCA CT(G CCG GAC T)CC ACC CGG CAG
110	110A005/s-110	F	Sporadic	c.794G>A	GCC AAA T**A**G AGA AGA
112	112A047/f-113	F	Familial	c.199A>T	TCC ATC **T**GA CCC AGG

112	112A065/f-112	F	Familial	c.199A>T	TCC ATC **T**GA CCC AGG
112	112A068/f-114	M	Familial	c.199A>T	TCC ATC **T**GA CCC AGG
112	112A147/f-128	F	Familial	c.199A>T	TCC ATC **T**GA CCC AGG
114	114A157/s-172	F	Sporadic	c.482delG	CGC CCT G(G)T TGG TAT
114	114A159/f-173	M	Familial	c.482delG	CGC CCT G(G)T TGG TAT
114	114A158/f-174	F	Familial	c.482delG	CGC CCT G(G)T TGG TAT
115	115A062/s-115	F	Sporadic	c.1268A>T	TTA CAG TTA] AAAAAAAAAAA
117	117A040/s-117	F	Sporadic	c.524-101_534del112 bp	ggtgaggctg(tcgggatataatgctcttggagtttaagactacaccaggccccttttggaggctccaagttaatccaaatttctcttaccatcctatttcttttgttccag/ATGGCTGCCAG) CAACAGGAAGG
118	118A051/s-118	M	Sporadic	c.57delG	GTC AAC GGG CG(G) CCA CTG CCG
119	119A107/s-119	M	Sporadic	c.1183+1G>A	ACTTCAACAG/**a**tgagccactgc

120	120A131/f-120	M	Familial	c.949C>T	TTG GGC **T**GA ACA GAC
122	122A023/s-122	F	Sporadic	c.1033-2A>G	ctctcctcac**g**g/CCCCCAGTCC
122	122A221/f-197	F	Familial	c.1033-2A>G	ctctcctcac**g**g/CCCCCAGTCC
124	124A141/s-124	F	Sporadic	c.771delG	CAG GTA TG(G) TTT TCT AAT
126	126A092/s-126	M	Sporadic	c.467G>A	GGA AGC T**A**G GGC ACC
127	127A123/s-127	F	Sporadic	c.480delT	ACC CGC CC(T) GGT TGG TAT CCG
128	128A200/s-190	F	Sporadic	c.141+18_+20delGCC	AATTCTGCAG/gtgatcctcccggc gcc(gcc)ccac
129	129A024/s-129	F	Sporadic	c.357+1G>A	CATACCAAGC/**a**taagttcattg
130	130A081/f-130	M	Familial	c.1032+6T>G	CTGCCTATGCAA/gtaag**g**gcgg ct
130	130A135/f-170	F	Familial	c.1032+6T>G	CTGCCTATGCAA/gtaag**g**gcgg ct
133	133A039/s-133	F	Sporadic	c.112delC	GGG GCC (C)GG CCG TGC
135	135A035/s-	F	Sporadic	c.122_123insGCGG	CCG TGC GA(GCGG)C ATT

	135				TCC
138	138A028/s-138	M	Sporadic	c.141+1G>T	AATTCTGCAG/**t**tgatcctcccg
140	140A195/s-185	M	Sporadic	c.1268A>T	TTA CAG TTA] AAAAAAAAAAA
141	141A009/s-141	F	Sporadic	c.401delA	GCT AGC GAA A(A)G CAA CAG ATG
141	141A010/f-140	M	Familial	c.401delA	GCT AGC GAA A(A)G CAA CAG ATG
143	143A077/s-143	F	Sporadic	c.1174_+6delACTTCA ACAGGTGAGC	GGGCACCTCGGGCACC(ACT TCAACAG/gtgagc)cactg
144	144A083/f-144	F	Familial	c.781C>T	TCT AAT **T**GA AGG GCC
144	144A089/f-167	M	Familial	c.781C>T	TCT AAT **T**GA AGG GCC
144	144A300/f-224	M	Familial	c.781C>T	TCT AAT **T**GA AGG GCC
147	147A017/f-147	M	Familial	c.204delC	ATC AGA CC(C) AGG GCA
147	147A059/f-146	M	Familial	c.204delC	ATC AGA CC(C) AGG GCA
147	147A061/f-149	M	Familial	c.204delC	ATC AGA CC(C) AGG GCA

147	147A064/f-148	F	Familial	c.204delC	ATC AGA CC(C) AGG GCA
150	150A018/s-150	F	Sporadic	c.179-185delATTACGAinsCTGAT	GGGCAGGT(CTGAT)GACTGGCTCCAT
150	150A106/f-151	M	Familial	c.179-185delATTACGAinsCTGAT	GGGCAGGT(CTGAT)GACTGGCTCCAT
152	152A114/s-152	F	Sporadic	c.141+2_+30delTGATCCTCCCGGCGCCGCCCCACTCGCCG	AATTCTGCAG/g(tgatcctcccggcgccgccccactcgccg)cccccgcggccc
153	153A075/s-153	F	Sporadic	c.949C>T	TTG GGC **T**GA ACA GAC
153	153A048/f-154	M	Familial	c.949C>T	TTG GGC **T**GA ACA GAC
155	155A102/s-155	M	Sporadic	c.332insG	GAG GGG G**G**TC TGT ACC
157	157A121/s-157	F	Sporadic	c.28C>T	GTG AAT **T**AG CTC GGT
160	160A091/s-160	F	Sporadic	c.607C>T	CAA ATG **T**GA CTT CAG
163	163A041/s-163	F	Sporadic	c.352-357+2delCCAAGCGT	CATA(CCAAGC/gt)aagttcattg

164	164A060/s-164	F	Sporadic	c.781C>T	TCT AAT **T**GA AGG GCC
168	168A014/s-168	F	Sporadic	c.365C>A	GTG TCA T**A**A ATA AAC
169	169A080/s-169	F	Sporadic	c.358-3_361delCAGGTGT	tttgatttg(cag/GTGT)CATCAA
170	170A174/s-184	F	Sporadic	c.781C>T	TCT AAT **T**GA AGG GCC
173	173A291/s-220	F	Sporadic	c.766-3C>G	cctgatttc**g**ag/GTATGGTTTT
185	185A164/s-180	F	Sporadic	c.1256delC	TGG C(C)A AGA TTA CAG TAA
189	189A257/s-209	M	Sporadic	c.631C>T	AAG CTG **T**AA AGA AAT
191	191A297/s-222	F	Sporadic	c.799A>T	TGG AGA **T**GA GAA GAA
227	227A302/s-227	M	Sporadic	c.795G>A	GCC AAA TG**A** AGA AGA
230	230A309/s-230	M	Sporadic	c.357+1G>A	CATACCAAGC/**a**taagttcattg
232	232A315/s-211	F	Sporadic	c.781C>T	TCT AAT **T**GA AGG GCC
100	1001A316/sU	M	Sporadic	c.949C>T	TTG GGC **T**GA ACA GAC

1	GA				
100 3	1003A318/sUGA	F	Sporadic	c.343delG	TGT ACC AAC (G)AT AAC ATA
100 4	1004A321/sUGA	M	Sporadic	c.1183+1G>A	CAACAG/**a**tgagcc
228	228A305/s-228	M	Sporadic	Deletion P1 promoter to exon 4	
100 2	1002A317/sUGA	F	Sporadic	Deletion exons 6 and 7	
161	161A100/s-161	F	Sporadic	WT1-PAX6; 3' extent unknown	
174	174A185/sw-191	F	Sporadic	WT1-PAX6; 3' extent unknown	
180	180A182/sw-190	F	Sporadic	WT1-PAX6; 3' extent unknown	
182	182A239/sw-201	F	Sporadic	WT1-PAX6; 3' extent unknown	
214	214A285/s060158	M	Parents Unknown	WT1-PAX6; 3' extent unknown	
219	219A261/s121996	F	Sporadic	WT1-PAX6; 3' extent unknown	
225	225A280/s725	F	Sporadic	WT1-PAX6; 3' extent	

	81			unknown
501	501A320/sUGA	F	Sporadic	Deletion of PAX6
121	121A049/s-121	F	Sporadic	No changes detected
162	162A122/s-162	F	Sporadic	c.766-12C>T
166	166A069/s-166	F	Sporadic	c.766-12C>T
190	190A305/501F	F	Sporadic	c.766-12C>T
231	231A312/s-231	F	Sporadic	No changes detected

Table 2.S.1. Demographic and sequence variant data for all 66 families included in the study. This includes the number of family members sequenced and affected, sex and full sequence variations.

Exon	5' flanking sequence	Exon size (bp)	3' flanking sequence	
0	…gcctgcctgagc[ACCCTCTTTT…	153	…CCGCTCCCAG/gtaaccgcccgg…	P_0, 5' UTR
1	…tgagagcgagcg[GTGCATTTGC…	216	…ATCAGCATAG/gtgtgctggctg…	P_1, 5' UTR
1'	…tgagagcgagcg[GTGCATTTGC…	503	…CCTCATAAAG/gtgagtccgctt…	5' UTR
2	…tctccttcccag/GAATCTGAGA…	188	…CCTCATAAAG/gtgagtccgctt…	5' UTR
3	…gttttgtcttag/GGGGAAGACT…	77	…GAGATTTCAG/gcaagttctgtg…	5' UTR
4	…tcttgctaacag/AGCCCCATAT…	61	…ATGCAGAACA/gtaagtgcctct…	Start ORF
alpha	…cgtcaatttatc[AGTAGGCTCC…	~95	…GGGGCTGCAG/gttggagatttt…	Pα, UTR
5	…ccctttcctcag/GTCACAGCGG…	131	…AATTCTGCAG/gtgatcctcccg…	Coding
5a	…tttgtgttatag/ACCCATGCAG…	42	…CAATCAAAAC/gtaagcttgtca…	Coding
6	…tcccctatgcag/GTGTCCAACG…	216	…CATACCAAGC/gtaagttcattg…	Coding
7	…tttgatttgcag/GTGTCATCAA…	166	…CCTACGCAAG/gtaaaacccaag…	Coding
8	…tttttgttccag/ATGGCTGCCA…	159	…CTGGAGAAAG/gtgatagagttt…	Coding
9	…ttactctttcag/AGTTTGAGAG…	83	…AAGAATACAG/gtaccgagagac…	Coding
10	…cctgatttccag/GTATGGTTTT…	151	…ACCACACCGG/gtaatttgaaat…	Coding
11	…tcttgccctcag/TTTCCTCCTT…	116	…GCCTATGCAA/gtaagtgcggct…	Coding
12	…ctctcctcacag/CCCCCAGTCC…	151	…ACTTCAACAG/gtgagccactgc…	Coding
13	…atccacttctag/GACTCATTTC…	1110	…TTTAACCTTA]tacagttttctt…	END ORF

Table 2.S.2. Intron and exon sequences. Shown in lower and upper cases, respectively. Donor and acceptor splice sites are indicated by a slash (/). The 5' ends of transcripts corresponding to the P_0, P_1, and P_α promoters are indicated with forward brackets. A reverse bracket denotes the inferred 3'-most end of the untranslated sequence of exon 13 based on ten independent transcripts obtained from eye tissues and encompasses three potential polyadenylation sites located at 573 bp, 800 bp, and 967 bp, respectively past the start of exon 13.

CHAPTER 3:

NEUROANATOMICAL ABNORMALITIES IN ADULT PAX6 DEFICIENT MICE[2]

[2] Bobilev, A.M., Hekmatyar, K. & Lauderdale, J.D. 2016. *Under review at Brain Structure and Function.*

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 brain development. 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 have identified structural abnormalities in fiber tracts and subcortical structures in the brain including corpus callosum, anterior and posterior commissures, as well as polymicrogyria, in persons with aniridia using magnetic resonance imaging (MRI). This study employed high resolution MRI to identify neuroanatomical differences in the adult heterozygous *Small Eye*^{Neu} mouse model of the disorder. Increases in grey were observed in PAX6$^{Sey\ Neu/+}$ mice relative to wild-type littermates in olfactory bulb, superior colliculus, inferior colliculus, periaqueductal grey, and throughout the cerebral and cerebellar cortex. White matter increases were observed in the dorsal hippocampal commissure, posterior corpus callosum forceps, and the superior and medial cerebellar peduncles. In the cerebral cortex, increases in volume overlap with areas known to be involved in sensorimotor, olfactory, and auditory processing. Volumetric increases could indicate an increase in cellular density or an expansion of cellular layers caused by abnormal organization or cellular migration. In comparison to human MRI studies, results of this study suggest that differences in the cerebellum, cortex, and corpus callosum are conserved effects of Pax6 mutations while abnormalities in structures such as the anterior commissure are unique to human cases.

3.1 Introduction

The development of the mammalian brain is directed by the precise and combinatorial effects of numerous gene products. Neuroanatomical structures and networks are directed and later maintained in the adult brain by the expression of many of these genes. One such gene is paired-box 6 (*PAX6* in humans, *Pax6* in rodents), which is expressed in the developing eye, brain, spinal cord, and pancreas, and exhibits regionalized expression in the adult (Walther and Gruss 1991; Stoykova and Gruss 1994; Grindley et al. 1995; Sander et al. 1997). PAX6 is a highly conserved transcriptional regulator that is critical for normal ocular, nasal, and neural development. During development, PAX6 is involved in brain patterning and regionalization as well as the formation of neural circuits, particularly in the forebrain (Stoykova and Gruss 1994; Stoykova et al. 1996; Mastick et al. 1997; Stoykova et al. 1997; Bishop et al. 2000; Osumi 2001; Simpson and Price 2002; Manuel and Price 2005). Currently, the neuroanatomical implications of reduced functional expression of PAX6 in the brain have yet to be fully resolved.

Homozygous loss-of-function mutations of PAX6 lead to embryonic lethality, while heterozygous loss-of-function mutations produce a distinct ocular phenotype in rodents known as *Small eye,* and are causal for a condition known as aniridia in humans (Glaser et al. 1990; Glaser et al. 1992; Hill et al. 1992; Ton et al. 1992; Fujiwara et al. 1994; Glaser et al. 1994; Numayama-Tsuruta et al. 2007). Aniridia is diagnosed at birth by iris hypoplasia, and *PAX6*-mediated aniridia makes up ≥80% of all known cases (Prosser and van Heyningen 1998; Bobilev et al. 2015). Although aniridia is primarily characterized by ocular defects, recent evidence has emerged attributing brain-related phenotypes to the disorder. Among these irregularities are deficits in auditory processing, olfactory dysfunction and deficient pituitary function, as well as increased functional brain connectivity in resting state networks as measured by functional

magnetic resonance imaging (fMRI) (Sisodiya et al. 2001; Bamiou et al. 2004a; Bamiou et al. 2007b; Pierce et al. 2014; Shimo et al. 2014). These phenotypic traits are likely a consequence of reduced *PAX6* function during the development of the brain and organization of neural circuitry.

Detailed investigation into the effects of *Pax6* haploinsufficiency in the adult brain can be accomplished using rodent models. One group has employed MRI to assess phenotypic changes in brain structure associated with conditional removal of *Pax6* from *Emx1*-expressing neurons in the developing cortex (Boretius et al. 2009). These mutant animals exhibited several neuroanatomical changes, including reduced whole brain and olfactory bulb volume, decreased cortical thickness and decreased white matter tract connectivity. Further, these mice demonstrated abnormalities in cortical layering and progenitor cell populations when PAX6 was conditionally knocked out in the cortex during early and late neurogenesis (Piñon et al. 2008; Boretius et al. 2009; Tuoc et al. 2009).

While these studies show some abnormalities consistent with human patients, the genetic construct is not representative of a patient mutation because it is knocked out in selective cells and does not represent the global consequences of *Pax6* heterozygous loss-of-function throughout the lifespan. The *Pax6* Neuherberg allele (Sey^{Neu}) consists of a splice-junction mutation at the intersection of exon 10 and intron 10 which ultimately leads to a premature termination codon (PTC) in the intronic sequence (Hill et al. 1991). The location and nature of this mutation putatively leads to haploinsufficient levels of PAX6 protein, and as such is similar to a majority of human *PAX6*-mediated aniridia cases (Vincent et al. 2003; Bobilev et al. 2015).

The current study sought to identify differences in neuroanatomical structures between live wild-type and *Pax6* haploinsufficient mice using high resolution structural magnetic resonance

imaging (MRI), analyzed with both region of interest measures and voxel-based morphometry (VBM). VBM is a statistically-based analytical method for performing voxel-based comparisons of grey or white matter visualized in MRI images between two groups of subjects (Wright et al. 1995; Wright et al. 1999; Ashburner and Friston 2000; Davatzikos et al. 2001). This method is widely used in human neuroimaging studies to investigate neuroanatomical correlates of both normal brain development and neurological disorders. In this approach, structural MRI data are converted into spatially normalized images for gray or white matter density. This is achieved by first segmenting the images into different components reflecting grey matter, white matter and cerebral spinal fluid, then smoothing the segmented images, followed by spatially transforming the images into a standard steotactic space. In this way, VBM corrects for interindividual variability in brain size and shape and permits the use of conventional Gaussian statistical techniques for assessing regionally specific changes in gray or white matter between population groups. This study employed VBM analysis to examine anatomical differences in vivo, precluding artifacts from histological tissue processing and providing a data set comparable to human studies of aniridia.

3.2 Methods

3.2.1 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 (Kim and Lauderdale 2006). 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 underwent 2D MRI imaging with manganese enhanced contrast injections. One mouse (male, $PAX6^{Sey\text{-}Neu/+}$) died before 3D image acquisition 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 3D MRI analyses.

3.2.2 Magnetic Resonance Imaging (MRI) Data Collection

This study employed high resolution MRI using a 7T Agilent system to acquire structural brain images using both 2D T1-weighted manganese enhanced MRI (MEMRI), 2D T2-weighted and 3D T2-weighted fast spin echo sequences. Mice were administered $MnCl_2$ contrast agent at a rate of 40mg/kg bodyweight for 3 consecutive days prior to T1-weighted image acquisition using T1-weighted spin echo imaging (TR/TE 500/12 msec, matrix 128 x 128, nt =8 with fov of 22 x 22) (Pautler et al. 1998; Lee et al. 2005). The 2D T2 and 3D T2 acquisition was performed on a separate day (2D: TR/TE 4600/35 msec, nt =16; 3D: TR/TE 400/44 msec, nt=4). MRI data were collected with 0.1718 mm x 0.1718 x 0.1718 mm resolution along three different directions (128 slices at 0.1718 mm).

3.2.3 MRI Data Analysis

Voxel-based morphometry (VBM) analysis was performed using Statistical Parametric Mapping software (SPM8; Wellcome Trust Centre for Neuroimaging, http://www.fil.ion.ucl.ac.uk/spm/)(Friston et al. 1995a; Friston et al. 1995b), run on a MATLAB platform (MATLAB Release 2015b, The Mathworks, Inc., Natick, Massachusetts, United States) following previously described methods (Ashburner and Friston 2000; Ashburner and Friston 2005) as adapted to mouse (spmmouse.org) (Sawiak et al. 2009a; Sawiak et al. 2009b). To

perform VBM, pixel resolution was adjusted to 1.718 x 1.718 x 1.718 mm. All 3D images were skull removed, aligned and co-registered with an adult C57BL/6J mouse brain template (Ma et al. 2005), which has a resolution of 700 microns. Coregistration was performed using the anterior commissure. Segmentations were performed on processed images to separate white matter, grey matter and cerebro-spinal fluid (CSF). Segmented grey and white matter images were smoothed with a 0.2mm FWHM (full width half maximum) isotropic Gaussian kernel and compared with two-sample F-tests. (False Discovery Rate (FDR) corrected $p<0.05$; cluster size of 100).

3.3 Results

VBM analysis revealed several anatomical regions demonstrating significant grey and white matter volume increases in *Pax6*$^{Sey\ Neu/+}$ mice compared to wild-type littermates. No significant decreases in volume of *Pax6*$^{Sey\ Neu/+}$ mice compared to wild-type were detected in either tissue type. Evaluation of grey matter volume presented significant bilateral increases at all axial levels of the brain (Figure 1a). Moving rostrally to caudally, significant changes were observed in the medial olfactory bulb (likely the anterior olfactory nucleus, slices +3.4 to +2.2), cerebral cortex, midbrain and cerebellum. In the cerebral cortex, changes were observed in the region of the motor cortex (slices +2.2 to +1.6), somatosensory cortex (slices +1.0 to -0.8) cingulate cortex (slice +1.0), hippocampal formation (slices -2.0 to -3.2), cingulate/retrosplenial area (slices -2.6 to -3.8), and auditory and visual cortices (slices -2.0 to -5.0). The majority of differences occurred adjacent to the corpus callosum and external capsule (slices -0.2 to -4.4). Changes in the midbrain were largely associated with the superior and inferior colliculi and periaqueductal grey (slices -3.8 to -5.0; and in lobules III, IV, and V of the cerebellum (slices -6.2 to -8.0).

White matter volume increases were seen in the posterior corpus callosum forceps, arbor vitae, and throughout the dorsal hippocampal commissure (slices -2.6 to -4.4, Figure 1b). In the cerebellum, increases in white matter were detected throughout the superior and middle cerebellar peduncles (slices -5.0 to -7.4, Figure 1b). Volumetric increases in white matter, which were observed in the dorsal hippocampal commissure as well as the superior and inferior cerebellar peduncles were consistent along the rostral-caudal axis throughout each fiber tract.

3.4 Discussion

The role of Pax6 in mammalian brain development has been extensively characterized through spatiotemporal expression studies and functional mutation models. However, the global effects of Pax6 mutations on adult brain structure have yet to be examined in Pax6 deficient mice. This study has identified several neuroanatomical changes in live heterozygous Pax6 deficient mice compared to their wild-type littermates that provide insights towards (i) the global effects of Pax6 haploinsufficiency on mammalian brain anatomy, (ii) conserved neuroanatomical abnormalities related to Pax6 mutations, and (iii) possible structural correlates of cognitive and sensory phenotypes in Pax6-mediated disorders in humans.

Heterozygous null mutations in *PAX6* are known to lead to neuroanatomical changes in the human brain. MRI studies have revealed that individuals with PAX6-mediated aniridia have structural changes in the brain that can include hypoplasia of the olfactory bulbs, hypoplasia of the anterior and posterior commissures, agenesis of the corpus callosum, and absence of the pineal gland (Sisodiya et al. 2001; Bamiou et al. 2004a; Bamiou et al. 2007b; Hanish et al. 2015). Group VBM analyses reduced grey matter volume in the occipital lobe and ventral cerebellum, and increased grey matter volume in frontal and medial temporal lobes (Free et al. 2003; Ellison-Wright et al. 2004). These imaging studies have also shown that there considerable phenotypic

variation between patients, particularly with regard to the presence or absence of the anterior and posterior commissures, volume of corpus callosum and the hypoplasia of the olfactory bulbs (Sisodiya et al. 2001; Thompson et al. 2004; Abouzeid et al. 2009). It is therefore likely that some changes in brain structure are direct consequences of loss-of-function mutations in *PAX6*, while some are individual-specific and could be due to properties of gene products arising from specific mutations or modifier effects.

The molecular genetic basis of neuroanatomical changes associated with mutations in *PAX6* is important for understanding brain-related changes in aniridia, as well as the role of PAX6 in neural development and maintenance. In addition to visual deficits, individuals with *PAX6*-mediated aniridia often have additional sensory and cognitive phenotypes that can include deficient auditory processing, olfactory processing, problems with sleep-wake cycles and changes in working memory (Sisodiya et al. 2001; Bamiou et al. 2004b; Thompson et al. 2004; Bamiou et al. 2007b; Bamiou et al. 2007a). *PAX6*-mutations have also been implicated in autism (Davis et al. 2008). Although some of these cognitive phenotypes can be directly attributed to changes in specific structures (e.g. reduced olfactory bulb or pineal gland), others are not easily explained by structural reductions in interhemsipheric fiber tracts, and suggest more widespread changes in connectivity. Consistent with this idea, we recently showed using functional magnetic resonance imaging (fMRI) that individuals with aniridia exhibit as a group increases in functional resting state connectivity and recruitment of atypical neuroanatomical regions in executive control, primary visual, and default mode networks (Pierce et al. 2014), suggesting widespread changes in the brain associated with *PAX6* haploinsufficiency.

A full understanding of the effects of *PAX6* haploinsufficiency on adult brain structure and function can be accomplished using rodent models, where neuroanatomical alterations in the

adult can be placed in the context of the role of Pax6 both in neural development and in the adult brain. Although previous studies have addressed the requirement of Pax6 in both the developing and adult rodent brain, the effect of *Pax6* haploinsufficiency has not been much studied. As a first step, we used high-resolution structural MRI and VBM to identify differences in neuroanatomical structures between live wild-type and *Pax6* haploinsufficient mice.

Our thesis going into this study was that the brain regions most likely to be affected in adult heterozygous null mutant animals were those of the posterior cortex (due to altered cortical development) and in regions of the adult brain where Pax6 expression is maintained (and presumably needed for proper neural function). In the developing rodent cerebral cortex, Pax6 is expressed in a gradient with its highest levels rostrally (Bishop et al. 2000; Mi et al. 2013). In the absence of Pax6, caudal and medial neocortical areas expand rostrally and laterally at the expense of rostral and lateral areas (Bishop et al. 2000; Bishop et al. 2002), demonstrating that Pax6 is required for neocortical arealization. In the case of heterozygous null mice, a 50% reduction in Pax6 levels in the developing cortex would necessarily result in a rostral-ward shift of Pax6 protein levels and a predicted concomitant expansion of more caudal and medial neocortical areas. This scenario predicts an expansion of visual and auditory areas and a concomitant change in the somatosensory areas. Examination of the changes detected by VBM support the general outlines of this model. The most significant changes in gray matter overlap with the somatosensory, auditory and visual areas in the cortical layers adjacent to the corpus callosum forceps/external capsule.

Significant gray matter changes were observed for the hippocampus. These changes may reflect a change in cellular density or location. We established by mRNA *in situ* hybridization that *Pax6* is expressed by cells in CA1, CA2 and CA3 and at relatively higher levels in the

dentate gyrus of the adult hippocampus (data not shown, see also Allen Brain Atlas), suggesting that *Pax6* may play a direct role in the development and/ or function of the hippocampus. Alternatively, the reduction in Pax6 during brain development may change the location and/or shape of the hippocampus, which would be captured in a VBM analysis. Consistent with this idea, conditional removal of *Pax6* during cortical development resulted in the hippocampus becoming rostrally extended and its volume increased relative to that of the forebrain and midbrain (Boretius et al. 2009). Consistent with this study, a relative gray matter increase in the hippocampus has been reported for persons with aniridia (Ellison-Wright et al. 2004).

Pax6 is required for normal white matter tract organization and interhemispheric connectivity. Both longitudinal and commissural tract formation are affected in the absence of *Pax6* during embryonic brain development (Warren and Price 1997; Mastick et al. 1997), and conditional removal of *Pax6* in the developing cortex results in a loss or significant reduction in the corpus callosum and anterior commissure and a rostral shift in the location of the ventral hippocampal commissure (Boretius et al. 2009). Additionally, as described above, persons with aniridia can exhibit reductions in the anterior and posterior commissures and corpus callosum.

The Pax6-haploinsufficinet mice used in the present study did exhibit changes in white matter tracts, but did not demonstrate a gross reduction in either the anterior or posterior commissures. The most significant changes in cortical tracts were observed in the posterior corpus callosum forceps and throughout the dorsal hippocampal commissure. As with the gray matter, these apparent increases in fiber tract thickness could result from increased fiber density and/or a relative change in position. Changes in white matter tracts were also observed in the cerebellum, particularly in the arbor vitae and the superior and middle cerebellar peduncles. To

our knowledge, this is the first report of white matter tract changes in the cerebellum associated with Pax6 haploinsufficiency.

In conclusion, this study has identified several abnormalities in discrete grey and white matter neural structures as a consequence of heterozygous *Pax6* mutations in the mouse. These findings, compared with human MRI studies, provide key insights into the conserved effects of Pax6 mutations in the brain, and implicate abnormalities in other structures as unique consequences in humans. Further, these results identify potential neuroanatomical correlates of functional neural processing abnormalities which remain poorly understood. In this way, we propose that specific cognitive and sensory processing deficits in aniridia may be explained using the Small eye Neu mouse model in functional studies of the brain.

3.5 References

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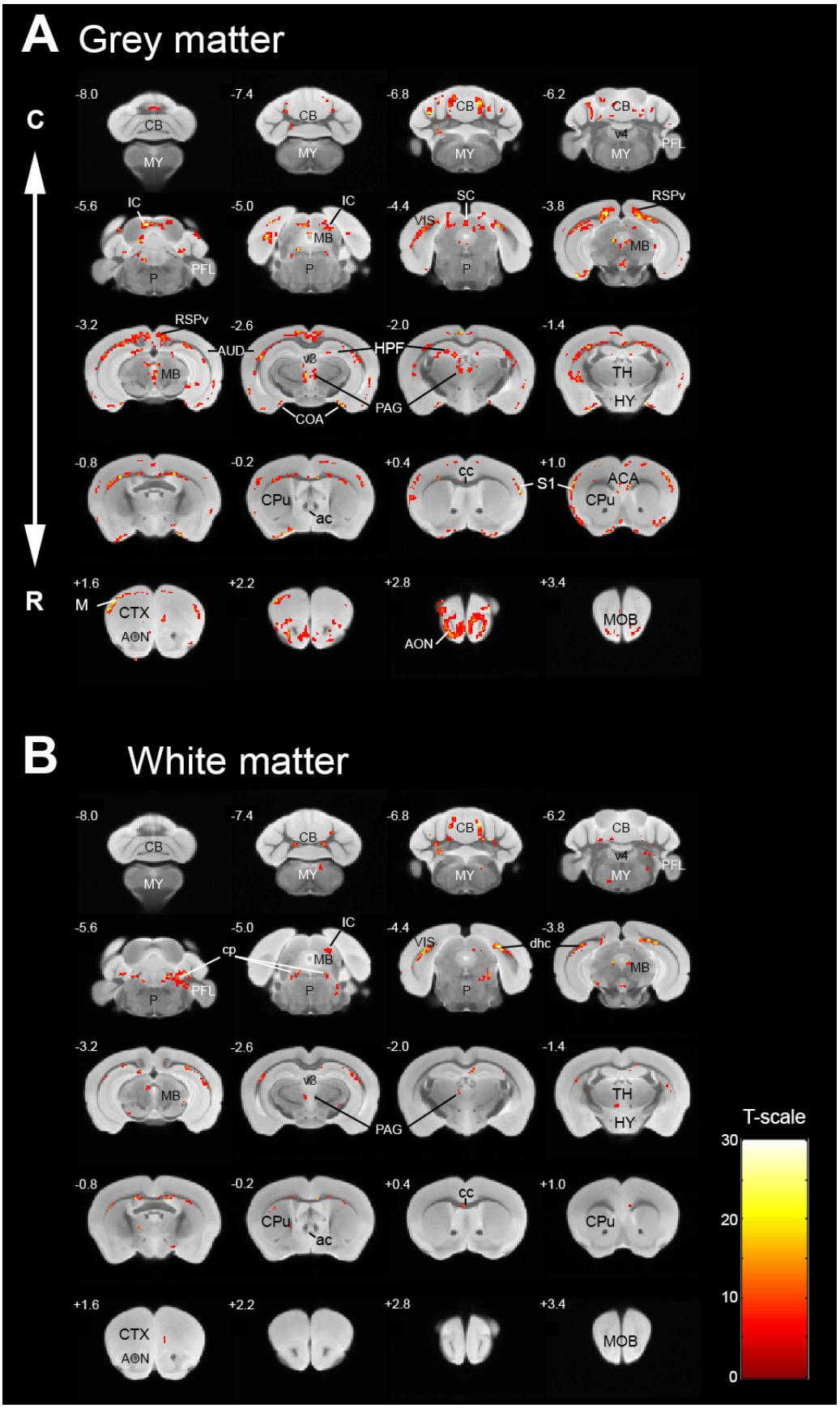
A Grey matter
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R
-8.0
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CPu
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ACA
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AON
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-6.2
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MY
PFL
-5.6
P
PFL
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IC
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VIS
dhc
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Figure 1. Voxel-Based Morphometry analysis reveals several anatomical regions demonstrating grey and white matter volume differences in PAX6 Sey Neu/+ mice compared to wild-type littermates. A. grey matter B. white matter. Coronal slices through adult *Pax6*$^{+/-}$ compared to wild-type littermate mouse brains. Slice numbers indicate their position in millimeters relative to bregma, designated as zero coordinate. Sections presented from caudal hindbrain (upper left) to rostral olfactory bulb (bottom right). Red pixels denote regions of statistically significant positive differences (FDR-corrected $p<.05$; cluster size of 100) of *Pax6*$^{+/-}$ animals (n = 8) compared to wild-type (n=8). Significance is shown with at statistic color scale, which corresponds to the level of the significance at the voxel level.

ACA, anterior cingulate area; ac, anterior commissure; AON, anterior olfactory nucleus; AUD, auditory area; cc, corpus callosum; COA, cortical amygdalar area; cp, cerebellar peduncles; CPu, caudate putamen (striatum); CTX, cerebral cortex; dhc, dorsal hippocampal commissure; HPF, hippocampal formation; HY, hypothalamus; M, motor area; MB, midbrain; MOB, main olfactory bulb; MY, medulla; P, pons; PFL, paraflocculus; RSPv, retrosplenial area, ventral part; S1, primary somatosensory cortex; TH, thalamus; v3, third ventricle

CHAPTER 4:

Early and late auditory information processing show opposing deviations in aniridia[3]

[3] Bobilev, A.M., Hudgens-Haney, M.E., Hamm, J.P., Oliver, W.T., McDowell, J.E., Lauderdale, J.D. & Clementz, B.A. 2016. *Under review at Cerebral Cortex.*

Abstract

Aniridia is a congenital disorder, predominantly caused by heterozygous mutations of the *PAX6* gene. While ocular defects have been extensively characterized in this population, brain-related anatomical and functional abnormalities are emerging as a prominent feature of the disorder. Previous work has determined that despite normal audiograms, individuals with aniridia frequently present with auditory processing deficits, attributed to hypoplasia of the anterior commissure and corpus callosum. The neurobiological basis of these impairments in aniridia remains poorly understood. This study provides direct assessment of neural activity related to auditory processing in aniridia. Subjects were presented with tones designed to elicit an auditory steady-state response (aSSR) at 22 Hz, 40 Hz, and 84 Hz, and infrequent broadband target tones to maintain attention during electroencephalography (EEG) recording. Persons with aniridia showed increased early cortical responses (P50 AEP) in response to all tones, and increased high-frequency oscillatory entrainment (84 Hz aSSR). In contrast, this group showed a decreased cortical integration response (P300 AEP to target tones) and reduced neural entrainment to cortical beta-band stimuli (22 Hz aSSR). Collectively, our results suggest that early, subcortical auditory processing is augmented in aniridia, while functional cortical integration of auditory information is deficient in this population.

4.1 Introduction

Auditory information processing is dependent on specialized function and connectivity in several areas of the brain, including finely tuned subcortical and cortical connections and functional integrative networks. Determining the neurophysiological causes of human auditory processing deficits presents a substantial challenge, as these deficits may result from either inappropriate development and/or maintenance of these complex neural circuits. A genetic disorder known as aniridia, which is predominantly caused by mutations in the *PAX6* gene, is associated with several sensory processing impairments including auditory discrimination abilities. Persons with aniridia have normal hearing, however the neural basis of auditory processing differences in this population is not well understood. Direct assessment of auditory processing via neuroimaging provides an opportunity to better understand cortical functioning in this disorder, as well as the effects of a candidate neurodevelopmental gene on functioning of the central auditory processing pathway.

Aniridia occurs in approximately 1.2 in 100,000 live births, and is primarily characterized by absence or hypoplasia of the iris (Grønskov et al., 2014; Jordan et al., 1992). Individuals with this disorder frequently exhibit secondary functional and progressive symptomatology affecting the eye (Hanson et al., 1993; Nelson, Spaeth, Nowinski, Margo, & Jackson; Nishida, Kinoshita, Ohashi, Kuwayama, & Yamamoto, 1995). In addition to ocular phenotypes, persons with aniridia often present with neural and neurocognitive abnormalities. Specifically, magnetic resonance imaging (MRI) studies have demonstrated structural brain abnormalities in persons with aniridia including hypoplasia of the anterior and posterior commissures, and agenesis of the corpus callosum (Bamiou et al., 2007; Free et al., 2003; Sisodiya et al., 2001). Voxel-based group analyses of MRI data in aniridia compared to healthy subjects have demonstrated reduced

grey matter volume in frontoparietal cortex, occipital lobe, and ventral cerebellum, and increased grey matter volume in striatum, insula, cerebellum, cingulate cortex, and medial temporal lobes (Ellison-Wright et al., 2004; Free et al., 2003; Yogarajah et al., 2016). These deviations in cortical grey matter volume could be attributable to dysregulated corticogenesis during development, and/or accelerated age-related cortical thinning (Barkovich, Guerrini, Kuzniecky, Jackson, & Dobyns, 2012; Yogarajah et al., 2016). Polymicrogyria (an excessive number of undersized gyri) has also been reported in the temporal lobe in a subset of patients, and is associated with disruptions in cortical migration (Mitchell et al., 2003).

Persons with aniridia also present with considerable deficits in central auditory processing tests despite normal audiograms, which has been attributed to abnormalities in inter-hemispheric transfer caused by the reduction in primary commissures carrying axonal projections between the temporal lobes (anterior commissure and corpus callosum) (Bamiou et al., 2004, 2007). In these studies, persons with aniridia showed abnormal auditory processing as measured by dichotic speech, pattern, and gaps in noise tests, which consistently presented with hypoplasia of the anterior commissure and/or corpus callosum in the population measured (Bamiou et al., 2004, 2007). A functional assessment of auditory processing circuits underlying these deficits in aniridia has yet to be published.

Aniridia is typically caused by heterozygous loss-of-function mutations of the *PAX6* gene (Bobilev et al., 2015a; Prosser & van Heyningen, 1998; Tzoulaki, White, & Hanson, 2005). The *PAX6* gene encodes a highly conserved transcription factor critical for normal brain development (Hanson et al., 1993; Simpson & Price, 2002). PAX6 is required for neuronal cell fate decision and appropriate migration during corticogenesis (Warren, 1999). Additionally, PAX6 plays a role in axon guidance and neural circuitry formation in both local and inter-hemispheric cortico-

cortical and cortico-thalamic networks (Hevner, Miyashita-Lin, & Rubenstein, 2002; Jones, Lopez-Bendito, Gruss, Stoykova, & Molnar, 2002; Mastick, Davis, Andrew, & Easter, 1997). In humans, the functional components of the auditory processing pathway in the brain overlap greatly with the neuronal subtypes and regional connectivity that are dependent on or modulated by PAX6 expression. The relationship between PAX6 dosage and auditory pathway integrity has not yet been directly experimentally determined.

Auditory information in the human brain is processed in the afferent auditory processing pathway, followed by top-down modulation and sensory integration networks (Ades et al., 1974; Webster, Popper, & Fay, 1992). The afferent auditory processing pathway begins with input from the cochlea to the cochlear nucleus in the brain stem. This information is then sent via the superior olivary nucleus and the inferior colliculus to the medial geniculate body of the thalamus, which in turn directs these signals to the primary auditory cortex. These afferent signals travel via ipsilateral and/or contralateral paths from the brain stem to the cortex. Sustained auditory stimulation that requires higher order discrimination predominately elicits contralateral cortical responses, and demonstrates a right hemisphere dominance (Ross, Herdman, & Pantev, 2005). Cortical auditory information is then subject to top-down modulation by cortico-cortical and corticofugal circuits, as well as to integration with other sensory and higher-order cortical information. Experimental recordings of auditory information processing in the brain typically represent midbrain collicular and thalamic activity 10-70ms post-stimulus onset, and cortical response after 40ms post-stimulus onset. Activity measured around 300ms post-stimulus onset represents cortical integration and modulatory circuit activity (Polich & Kok, 1995). Anatomical abnormalities in interhemispheric structures such as the anterior commissure could disrupt auditory information processing at these higher levels, while polymicrogyria observed in the

superior temporal lobe could be indicative of processing disruptions in the cortical component of the auditory pathway. However, these anatomical abnormalities are not sufficient to identify the functional and physiological correlates of auditory processing.

Electroencephalography (EEG) is a direct measure of brain activity with high temporal precision, which makes it ideal for studying auditory processing dynamics in humans. Previous studies suggest that discrete components of the auditory information relay are dissociable by temporal and frequency-specific characteristics of EEG data. Presentation of auditory stimuli during EEG recording elicits a characteristic auditory evoked potential (AEP) waveform, which temporally correlates with early brainstem, midbrain, and cortical registration of sounds (Moore, 1987; T.W. Picton, Hillyard, Krausz, & Galambos, 1974). Stimuli presented binaurally typically elicit AEPs with higher amplitude, particularly in early brain-stem associated components, than monaural stimuli, though no change is evident in response latency (Ainslie & Boston, 1980). Auditory stimuli can be presented at specific driving frequencies to induce entrainment of neural ensembles with differential preference to frequency-specific information encoding, known as the auditory steady-state response (aSSR) (John & Picton, 2000; Pantev et al., 1995; Rees, Green, & Kay, 1986; Romani, Williamson, & Kaufman, 1982; Zhang, Peng, Zhang, & Hu, 2013). These frequency-specific neural ensembles are associated with different functional aspects of auditory processing: beta band activity has been implicated in top-down modulation and integration of cortical auditory information, low gamma band activity has been associated with cortical afferent auditory encoding, and high gamma band activity has been associated with both local auditory cortical (Hamm, Gilmore, Picchetti, Sponheim, & Clementz, 2011) and subcortical sources (Fontolan, Morillon, Liegeois-Chauvel, & Giraud, 2014; Killian & Buffalo, 2014; Mäkelä &

Hari, 1987). Further, stimulus density in aSSR paradigms positively correlates with the magnitude of the N100 component of the AEP in healthy persons (Hamm et al., 2011).

The current study employed such an aSSR paradigm in pursuit of identifying the fine temporal dynamics of auditory processing disruptions in aniridia. Such information, when combined with anatomical deficits, could be used to infer the circuit level pathophysiology of the disorder. It is likely that the behavioral auditory processing deficits are consequences of several anatomical and functional abnormalities in the auditory processing stream, and cannot be explained by reduced commissural connections alone. We propose that deficits in auditory processing tasks described by previous studies likely originate from early auditory processing impairments, and are further disrupted at the level of cortical processing or integration. To elucidate early and late disruptions in auditory information transfer throughout the auditory processing pathway and frequency-specific processes, we examined both the AEP and aSSR in persons with aniridia relative to healthy comparison subjects. To specifically assess cortical integration abnormalities, the P300 response to targets in an "oddball" task were examined. Stimuli were presented both monaurally and binaurally to test whether disruptions in the auditory pathway were specific to left, right, or integrative signal processing.

4.2 Materials and Methods

Participants

Seventeen individuals with aniridia and 16 healthy comparison individuals participated in the current study. Data from one participant with aniridia was excluded from analyses (due to significant artifact in the majority of trials). The 16 individuals with aniridia (10 females, 4 left-handed individuals, mean age =39.2 years, SD=15.6) and 16 healthy comparison individuals (10 females, 4 left-handed individuals, mean age= 35.1 years, SD=13.3) were included in the

analyses (Table 1). Healthy comparison subjects were recruited through flyers posted in the community. Participants with aniridia were recruited through the Aniridia Foundation International Conference held in 2011 in Athens, Georgia, and had been clinically diagnosed with aniridia. Exonic sequencing of the *PAX6* gene (11p13) (OMIM: 607108) was conducted for ten of the participants with aniridia at the University of Georgia as previously described (Bobilev et al., 2015b). All mutations have been submitted to the Human *PAX6* Allelic Variant Database (http://lsdb.hgu.mrc.ac.uk/home.php?select_db=PAX6) as part of a previous genotype identification study and are reported in Table 1. Six participants did not have confirmed PAX6 mutations at the time of the study because they did not participate in the genotyping study. After written informed consent was obtained, all participants completed an EEG session in which an auditory paradigm was presented. All activities were approved by the Institutional Review Board of the University of Georgia prior to subject recruitment and data collection.

Stimuli

Stimuli were presented for 1500ms binaurally and monaurally (left ear or right ear) through Etymotic insert earphones (Etymotic Research, Elk Grove, IL, United States) at 76 dB SPL with an average inter-stimulus interval (ISI) of 2 seconds (randomly jittered 1800-2200ms). "Standard" stimuli consisted of broadband white noise sinusoidally amplitude modulated at one of three driving frequencies (22Hz, 40Hz and 84Hz) and "target" stimuli were unmodulated broadband white noise (500-1500Hz). Stimuli were presented in a block design (9 blocks: each block contained one driving frequency and presentation condition) while participants sat in a dark room with eyes open and were instructed to fixate on a small cross on a computer monitor. Each block consisted of 80 trials, 85% "standard" tones and 15% "target" tones. Participants were instructed to make a button press with both hands to target tones to maintain task attention.

Electroencephalography (EEG) data acquisition

EEG data were recorded vertex-referenced with a 256-sensor Geodesic Sensor Net and NetAmps 200 amplifiers (Electrical Geodesics Inc, Eugene OR, United States). Impedance of sensors was kept below 50kΩ and data were acquired at a sampling rate of 500Hz with an analog filter bandpass of 0.1-200Hz.

Data processing

Sensors located on the neck and face were excluded, leaving a 211-sensor configuration for data analysis. Raw data were inspected for bad sensors, which were identified and interpolated (≤5% of sensors for each participant) via spherical spline interpolation (BESA Software 5.0; MEGIS Software, Grafelfing, Germany). Data were converted to an average reference montage and filtered (.5-100Hz, zero phase, rolloff: 12 and 48 dB/octave respectively; notch filter 60Hz, 2Hz width). Blink, eye movement, and cardiac artifacts were identified via Independent Component Analysis and removed from the data using EEG Lab (Delorme & Makeig, 2004) on a MATLAB software platform (Release 2015b, The Mathworks, Inc., Natick, Massachusetts, United States). Data epochs for standard and target trials were segmented 750ms before stimulus onset to 2250ms after stimulus onset, and epochs with activity >120 microvolts at any sensor were eliminated prior to analysis.

AEP Data Analysis

Standard and target data epochs were averaged for each subject for each presentation block (presentation condition (binaural, left, right) and frequency (22 Hz, 40 Hz, 84 Hz)). To evaluate early signal processing characteristics, averages were reduced to -50 pre- to 500ms post-stimulus onset to generate AEPs for each subject. To accurately capture the shared variance in the spatial distribution of the AEP, a spatial principle component analysis (PCA) was

implemented using the ERP PCA Toolkit in MATLAB (Dien, 2012). PCA reduces data dimensionally to maximize the signal to noise ratio and, by replacing a group of variables with a linear combination of those variables, reduces redundancy of information while retaining as much data variance as possible, and best explains the variance across all measures. For each of the groups individually, all participants' AEP data were concatenated using sensors as variables and time points as observations, and a PCA was computed with Promax (oblique) vector rotation and Kaiser normalization (Dien, Khoe, & Mangun, 2007). This was carried out for standards and targets separately. Scree tests for each of the groups identified one component for standards and two components for targets. The spatial distribution of these component weights and amount of variance accounted for were nearly the same for both groups, so the same procedure was carried out again including all participants for standard and target PCAs. Data for individual subjects were converted to a single virtual sensor representing the AEP for each subject for each of the PCA components by computing the dot product of the component weights and the 211-channel data at each timepoint. A 3-way ANOVA (group (aniridia, healthy) x condition (binaural, left, right) x frequency (22 Hz, 40 Hz, 84 Hz)) was calculated for each 10ms time bin for standard stimuli AEPs. For target stimuli, in order to retain a sufficient number of trials for each participant, data from all stimulus conditions and frequencies were combined and compared between groups with a 1-way ANOVA for each component. To control for increased family-wise error rate due to multiple comparisons, a clustering method was used to take account of the non-independence of data from adjacent time bins (Monte Carlo simulations calculated using AlphaSim) (Cox, 1996; Forman et al., 1995). Time bin clusters were considered significant at overall family-wise $\alpha < .005$ if at least three adjacent 10ms bins were significant $p < .05$.

aSSR Data Analysis

To assess the neural response at each driving frequency, a Fast Fourier Transform (FFT) was applied to each subject's evoked response at each channel (-750 pre- to 2250 post-stimulus onset; 500ms sliding hanning window, 2ms steps; resulting power values were log transformed for normalization). A 2-way ANOVA (group (aniridia, healthy) x condition (binaural, left, right)) was conducted at each sensor for power at the respective driving frequency of each stimulus presentation. To assess entrainment-specific activity at each driving frequency, a spatial PCA was computed on all subjects for each frequency and two components were retained (verified by scree test; see AEP data analysis). The first and second components of the spatial PCA for each group individually reflected those derived from all subjects, although eigenvalue and rank order differed. For this reason, data for each subject and frequency were weighted by the spatial PCA solutions from the top two components (from the "all subjects" PCA), as for AEPs, but then a single virtual sensor was computed by creating an eigenvalue-weighted average between the two components. A virtual sensor was then computed for each subject reflecting these weights, and a one-way ANOVA was calculated for each 10ms time bin at each frequency. The clustering threshold method described for AEPs was implemented for the aSSR data, with time bin clusters considered significant at overall family-wise $\alpha < .001$ if at least four adjacent 10ms bins were significant $p < .05$.

4.3 Results

Significant differences in components of both the AEP and aSSR were found between aniridia and healthy subjects. Analyses of standard and target AEPs reveal differences in the amplitude of the response in specific temporal components of the signal, and analyses of aSSR demonstrate frequency-specific changes in entrainment to the driving frequencies.

AEP Findings

For standard stimuli, the first and only component reflected a typical auditory evoked response suggestive of temporal and frontal lobe generators. A group main effect was found from 40-70ms post-stimulus onset (F=12.64, p=.006; Fig 1), reflecting an increase in the P50 component of the AEP response in aniridia relative to healthy comparisons. Main effects for condition from 40-80ms (F=8.91, p=.005) and 150-230ms post-stimulus onset (F=14.4, p<.001) were present in AEPs, demonstrating an increase in the amplitude of the P50 and P200 components for both groups in response to binaural stimulus presentation relative to monaural, consistent with previous reports. No two-way or three-way interactions were observed in the standard AEP data, indicating that aural presentation and stimulus frequency did not differently affect the standard AEPs of aniridia and healthy persons. For target stimuli, the first AEP component reflected a posterior distribution similar to a typical P3b like response. Here, a significant time bin cluster was found in the first PCA component from 230-260ms post-stimulus onset (F=4.38, p=.046; Fig 1), showing an increase in aniridia response magnitude relative to healthy. The second PCA component had an anterior distribution similar to that of early-latency auditory evoked responses and, importantly, P3a. Here, the aniridia group showed increased amplitude as compared to healthy30-70ms (F=15.94, p=.007; Fig 1) and 170-220ms (F= 5.72, p=.031; Fig 1) post-stimulus onset. From 320-400ms (F=7.135, p=.020; Fig1), aniridia showed lower amplitude than healthy.

aSSR Findings

Typical aSSR entrainment to each driving frequency was observed in both groups in each frequency presentation (Fig 2). Group by aural condition ANOVAs computed separately for each sensor and frequency revealed no significant interactions. To increase the signal-to-noise

ratio for analysis of driving frequency entrainment and PCA analysis, each subject's data were combined over aural conditions for each presentation frequency and FFT was re-calculated. Although both groups demonstrated entrainment to the respective driving frequency in all three conditions, the magnitude of this response varied considerably between groups depending on which frequency was presented (Fig 3). In the 22 Hz condition, the aniridia group demonstrated significantly lower power at the driving frequency 660-780ms post-stimulus onset (F=4.604, p=.0405) and tended to have reduced power throughout the duration of the entrainment period. In the 40 Hz condition, no significant group differences at the driving frequency were detected. In the 84 Hz condition, aniridia subjects showed a significant increase relative to healthy in the entrainment period 50-140ms (F=5.102, p=.0322) and 700-920ms (F=5.707, p=.0267) post-stimulus onset, and tended to have increased power throughout the entrainment period.

4.4 Discussion

This study provides the first examination of functional auditory processing in aniridia measured by EEG. While auditory processing deficits in aniridia have previously been established by patient report and behavioral auditory tasks, the current study directly examined the neural basis. Results indicate that persons with aniridia have increased activity during early auditory stimulus recognition to all stimuli and decreased activity in late cortical responses representative of cortical integration networks. Data also revealed frequency-specific increases and decreases in entrainment to an auditory steady-state paradigm in high gamma and beta frequencies, respectively. These findings may reflect augmentations in subcortical and early sensory cortical stimulus registration and concomitant decreases in top-down or sensory integration processes in the cortex. Dissociable functional components of the auditory processing pathway may be disrupted in aniridia.

The increase in activity at P50 in the AEP and increased power to 84Hz in the aSSR in aniridia are both consistent with augmented neural activity in cortical (Hamm et al., 2011; Roß, Borgmann, Draganova, Roberts, & Pantev, 2000) or subcortical (Terence W. Picton, John, Dimitrijevic, & Purcell, 2003) auditory processing (Fig 1, Fig 3). Collectively, our results indicate a hyper-activation of neural populations during early registration of an auditory stimulus. These increases are not likely due to the neuroanatomical abnormalities previously associated with behavioral deficits, as subcortical structures involved in auditory processing transmit information independent of the anterior commissure. Instead, subcortical hyper-activation could be a consequence of an increase of cell numbers in these areas, or a decrease in the refined specific tuning of cellular populations. High gamma oscillations show early cortical sources (Hamm et al., 2011) and may arise from interneuron-pyramidal neuron circuits in granular layers. It is also possible that the increase in subcortical activity is an adaptive response to decreased or nonspecific efferent auditory inputs from the cortex. Downstream, this hyper-activation could lead to reduced discrimination of stimulus characteristics via decreases in gain control, ultimately contributing to behavioral-level impairments of auditory discrimination such as those seen in the gaps in noise test (Bamiou et al., 2007).

Cortical gamma (40 Hz) aSSR in aniridia is virtually indistinguishable from that observed in healthy comparison subjects (Fig 3). Additionally, the amplitude of the N100 in the AEP showed no significant deviations from healthy. This indicates that, despite the early augmented P50 response, neural populations in auditory cortex that receive initial cortical inputs may be functionally preserved. It is possible that neural populations responsible for early cortical processing, that generally display oscillations in the gamma band, are sufficient for normal cortical registration, or that compensatory mechanisms exist which support normal gamma band

entrainment. Beta activity (22 Hz) on the other hand, demonstrates a marked reduction in aSSR entrainment (Fig 3), presenting a higher-order disruption in auditory information processing in aniridia. This is consistent with reduced AEP amplitude to target tones after 300ms post-stimulus onset (Fig 1), indicative of deficient sensory integration activity. Beta band activity (12-30 Hz) has previously been associated with cortico-cortical communication, specifically in longer-range modulation of auditory cortex and sensory integration systems in the temporal lobe (Arnal & Giraud, 2012; Fontolan et al., 2014; Kayser & Logothetis, 2009). These cortical networks contribute to interhemispheric fiber tracts, so the decreased aSSR at 22 Hz may be caused by reductions in the anterior commissure or corpus callosum previously associated with behavioral deficits. Alternatively, the neural populations responsible for driving beta band activity may themselves be poorly connected or globally reduced. The decrease in 22 Hz entrainment likely represents a neurophysiological substrate of impaired higher-order processing that contributes to deficits in complex auditory perception reported in previous studies. The abnormalities reported here in aniridia could be a direct or ancillary consequence of mutations in the *PAX6* gene.

During development of the mammalian nervous system, PAX6 plays a demonstrable role in neural patterning and regionalization, particularly in the forebrain (Manuel & Price, 2005; A Stoykova, Fritsch, Walther, & Gruss, 1996; A Stoykova, Gotz, Gruss, & Price, 1997; Anastassia Stoykova, Treichel, Hallonet, & Gruss, 2000). At the cellular level, previous studies have presented evidence for a role of PAX6 in cortical neural progenitor cell division and initial cell fate decisions during early brain development, as well as maintenance of neuron subtypes (Asami et al., 2011; A Stoykova & Gruss, 1994). In humans, PAX6 has been implicated in cell fate decisions for both pyramidal cortical cells (projection neurons) and interneuron

differentiation (Mo & Zecevic, 2008). Not only the presence of PAX6, but the precise dosage of the protein is critical for normal corticogenesis. When PAX6 is conditionally knocked out in corticogenesis, an expansion of the deep layers of the cortex and reduction in superficial layers is observed. Conversely, when PAX6 is overexpressed in the developing cortex, an expansion of the superficial layers and reduction of the deep layers is seen. This dosage-dependence is likely a result of the role of PAX6 in maintaining progenitor cell fates. Additionally, PAX6 plays a role in axon guidance and neural circuitry formation in both local and inter-hemispheric cortico-cortical and cortico-thalamic networks (Hevner et al., 2002; Jones et al., 2002; Mastick et al., 1997). Persons with aniridia have a significant reduction in functional PAX6 as a consequence of loss-of-function mutations, therefore cortical development, organization, or connectivity may be altered. Should superficial layers of the auditory cortex in aniridia be affected in this way, the reduction in long-range activity represented by 22 Hz in this study would be consistent with a reduction of relevant cortical populations, while local networks preferential to gamma band entrainment would remain unaffected. Further, if cortico-thalamic components of the efferent auditory processing pathway are reduced in aniridia, subcortical activity may be upregulated in response to decreased top-down modulation. Future work should investigate the electrophysiological dynamics of frequency-specific information processing in model organisms of PAX6 mutations, as well as functional anatomical correlates in these models.

The findings presented here suggest that top-down modulation of auditory processing is deficient in aniridia, while early stimulus registration and local a1 or subcortical generators are normal and/or augmented in this population. Mutations in the PAX6 gene are responsible for the majority of occurrences of aniridia and, as such, suggests a potential role for this gene in the normal development and maintenance of the human auditory processing pathway.

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Aniridia Subjects				Healthy Subjects		
Gender	**Age**	**Handedness**	***PAX6* Mutation**	**Gender**	**Age**	**Handedness**
F	66	A	c.481delG	F	44	R
F	34	R	c.481delG	M	33	R
M	38	R	c.481delG	M	20	R
F	35	R	not confirmed	M	21	R
F	19	A	c.771delG	F	22	L
M	58	R	c.1032+6T>G	F	51	R
M	18	R	not confirmed	F	49	R
M	47	R	not confirmed	M	50	R
F	53	R	not confirmed	F	49	R
M	46	L	c.204delC	F	28	L
F	24	L	c.204delC	M	25	R
F	25	L	not confirmed	F	23	L
F	60	A	c.799A>T	M	25	R
M	20	R	c.204delC	F	47	L
F	34	L	not confirmed	F	54	R
F	51	R	c.766-3C>G	F	20	R

Table 1: Subject demographics. The gender (M=male, F=female), age, handedness (R=right handed, L=left handed, A=ambidextrous), and mutation of *PAX6* (in aniridia subjects, if available) are described for each participant in the study.

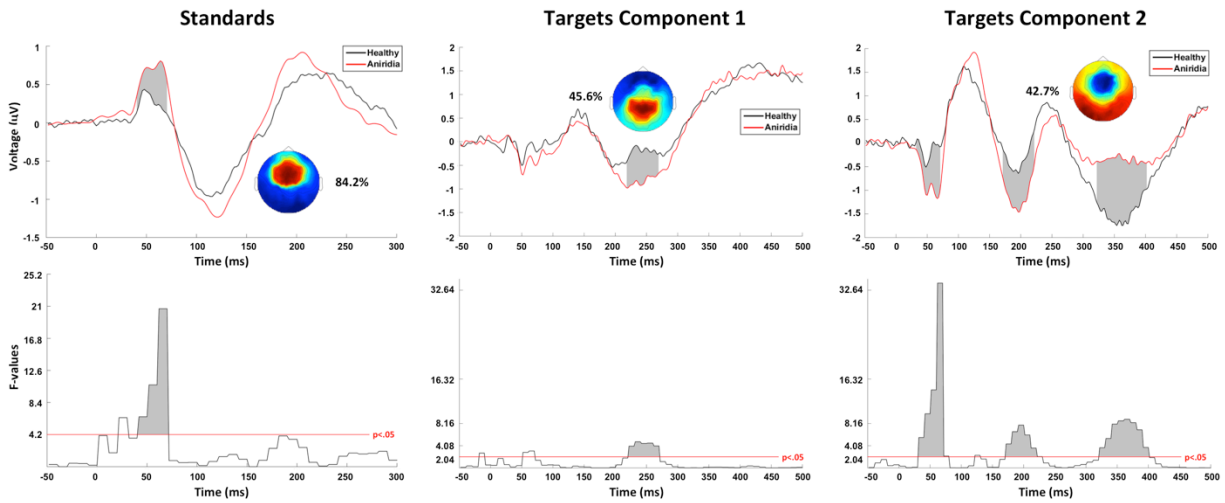

Figure 1: Standard and target AEPs. Principal components analysis weighted waveforms for each group for standards (left) and targets (middle and right). The upper panels show time (in ms; 0 represents stimulus onset) on the x axis and amplitude of response (in μV) on the y axis, with topographies of spatial PCA weights (top-down view) and the percent of total variance each component accounts for. The lower plots display *F* values for main effect of group (aniridia vs. healthy) for each 10ms time bin. Time bin clusters were significant at $p < .005$ if three consecutive time bins were significant at $p < .05$ (indicated by the horizontal red line), and are shaded in gray.

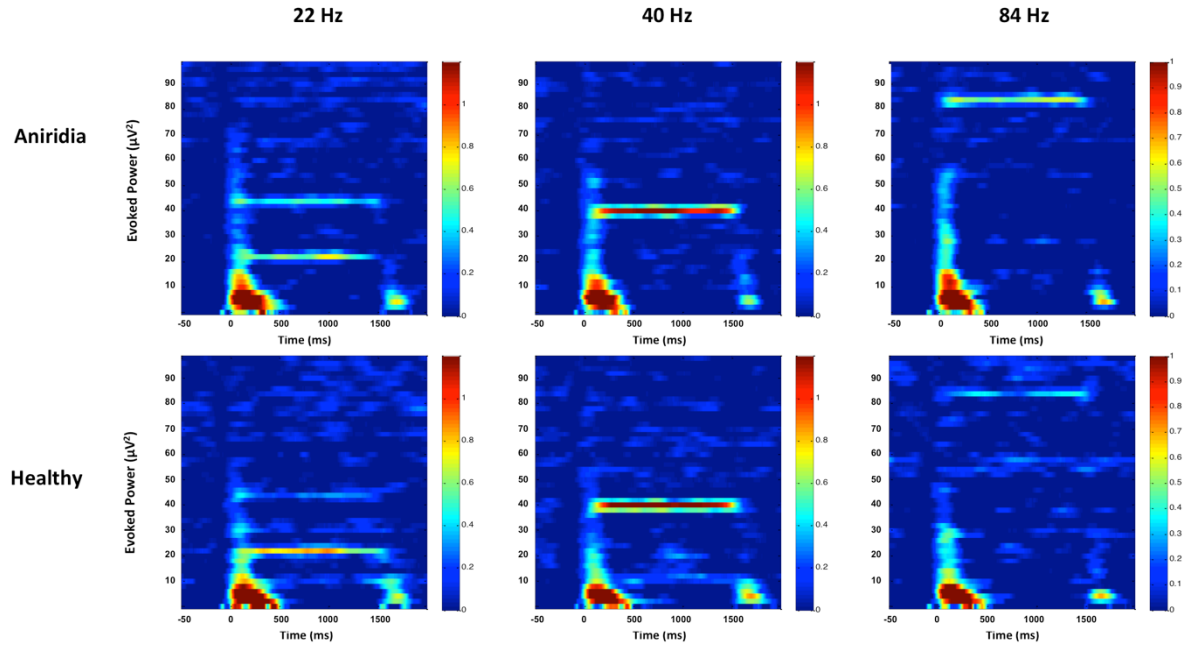

Figure 2: Evoked power for each driving frequency. Time-frequency plots show aniridia (top) and healthy (bottom) evoked power from 0 to 100 Hz (2 Hz steps) over time (-500 pre- to 2000ms post-stimulus onset; 0 represents stimulus onset) for 22 Hz, 40 Hz, and 84 Hz stimulus presentations. Color scale indicates baseline-subtracted power values in μV^2 from low (blue) to high (red). Data represents average over all 211 sensors.

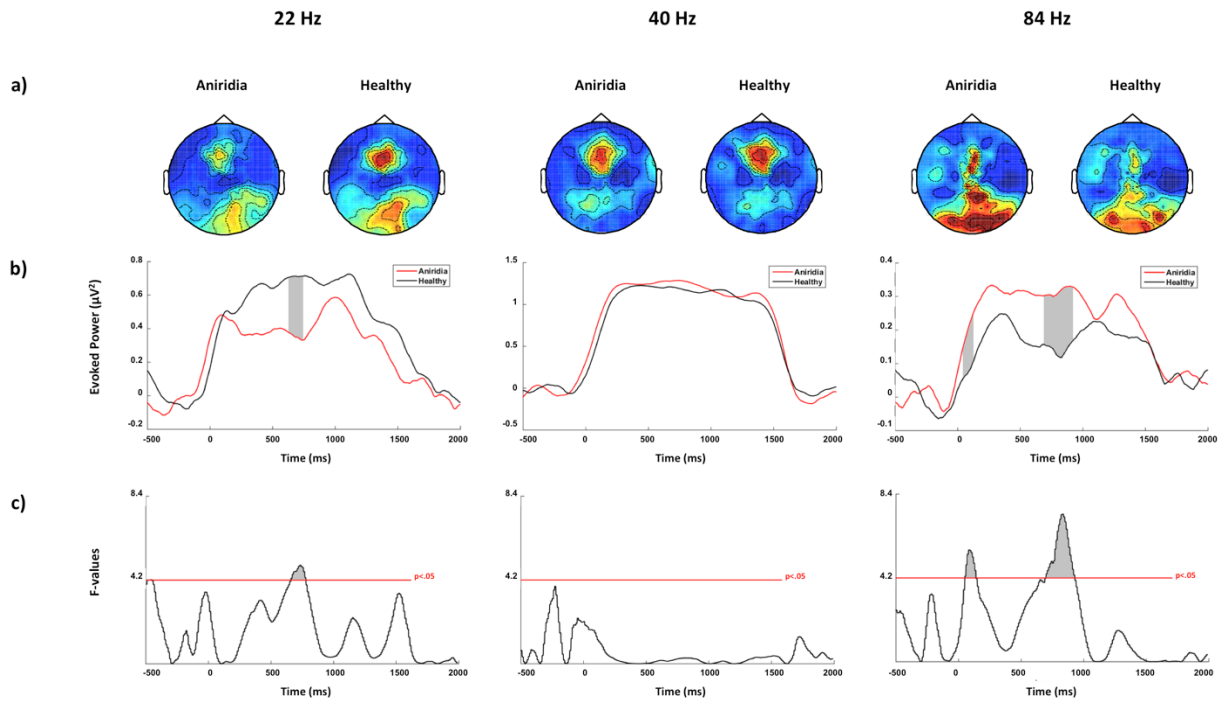

Figure 3: aSSR entrainment at each driving frequency. (A) Principal components analysis (PCA) weighted topographies by group of evoked power at the driving frequencies. Topographies show the spatial distribution (top-down view) of the top two spatial PCA components-weighted power values (blue=low, red=high) proportionately combined, averaged over the entrainment period (0 to 1500ms post-stimulus onset). (B) PCA weighted waveforms by group of evoked power at each frequency. (C) *F* values for main effect of group for each 10ms time bin. Time bin clusters were significant at $p < .001$ if four consecutive time bins were significant at $p < .05$ (indicated by the horizontal red line), and are shaded in gray.

CHAPTER 5:

DISCUSSION AND CONCLUSIONS

5.1 Understanding *PAX6*-mediated aniridia and the brain

5.1.1 A molecular and cellular perspective

Understanding the effects of various PAX6 mutations on brain structure and function demands a critical evaluation of molecular interactions and cellular processes governing DNA, RNA and protein. PAX6 is a large gene with four alternative start sites and three promoters, as well as a large number of regulatory elements that all contribute to the spatiotemporal context and dosage of PAX6 transcription. These transcripts are subject to RNA-surveillance mechanisms and alternative splicing preceding protein translation. These transcripts also encode four distinct protein domains with different functional roles. Therefore, the location and nature of mutations can have profoundly different effects on the generation of PAX6 products and the downstream interactions of these products in the cell.

The majority of molecular genomic mutations of PAX6 are caused by substitutions, small insertions and/or deletions (1 to 10,000 bp), or large deletions of nucleotide base pairs at the PAX6 locus (Weber et al., 2002). Occasionally, substitutions of a single nucleotide can be classified as "missense" mutations, meaning that the mutation changed the amino acid defined by the codon in which it occurs from one to another. Similarly, small insertions or deletions can be "in-frame," meaning that they do not affect the open reading frame (ORF) of PAX6 but rather add or subtract one or more amino acid codons. Missense and in-frame mutations likely do not affect transcription or translation of gene products, and the change in amino acid sequence may

have little to no effect on protein function. Alternatively, these changes sometimes disrupt protein folding or the functional activity of protein domains. The most dramatic example of this is seen in cases of missense mutations that lead to a run-on in the ORF.

Mutations that do affect the ORF of PAX6 can be classified according to their effect on mRNA: (i) causal for premature termination codons (PTCs), (ii) frameshifting, or (iii) splice junction disruptions. PTCs are often a result of single nucleotide substitutions that change a codon from an amino acid to a stop codon, which is reflected in the mRNA sequence and would produce a truncated protein if translated. Frameshift mutations alter a much larger portion of the ORF and mRNA sequence, shifting the codon sequences for many amino acids. In addition, frameshifts often result in inappropriate inclusion of intronic sequences in the ORF, leading to disruption of splice junctions and introduction of PTCs. Mutations that affect splicing alter PAX6 gene products at the level of mRNA processing, and can lead to inclusion of introns, exon skipping or cryptic splice site utilization (Krawczak, Reiss, & Cooper, 1992). Cryptic splice sites represent a molecular genomic "backup" that are repressed by nearby stronger splice sites, but can efficiently recruit spliceosome machinery towards appropriate mRNA splicing when a mutation directly affects the main site (Kapustin et al., 2011; Newmann, 1994; Padgett, Grabowski, Konarska, Seiler, & Sharp, 1986). Therefore, the effects of splice-junction mutations can range from small negligible alterations in protein conformation to non-functional protein products. Putative splice-junction mutations should be individually evaluated in the context of cryptic and alternative splice sites, and future work on PAX6 should apply new approaches towards identifying these sites with tools such as the cryptic splice site finder and observation of individual genomic sequences (Kapustin et al., 2011; Krawczak et al., 1992).

Mutations that occur in intronic, 5' or 3' sequences and are associated with a phenotype are assumed to significantly disrupt regulatory elements. In these cases, mutations may selectively prevent binding of upstream activators or repressors, or recruit inappropriate regulatory factors. Alternatively, mutations in regulatory elements may only affect sequences necessary for alternative splicing during nuclear RNA processing (Robberson, Cote, & Berget, 1990; Ward & Cooper, 2010). Because regulatory element necessity varies depending on the spatial and temporal cellular context, it is also possible that mutations in regulatory sequences have unique or restricted effects on PAX6 transcription. Mutations in exonic sequences also play a role in splice site selection, suggesting that missense or in-frame insertions or deletions that are otherwise considered less severe could have additional effects on mRNA (Reed & Maniatis, 1986).

The effect of PTC-causing mutations on PAX6 proteins depends largely on the location of the mutation and the susceptibility of the transcript to RNA-surveillance mechanisms. If transcripts harboring a PTC remain unmonitored by RNA surveillance and are translated, truncated proteins will be produced. In this case, the location of the PTC in the ORF of PAX6 is of critical importance: mutations affecting the coding sequence of the paired domain will result in non-functional proteins and likely result in haploinsufficiency of canonical PAX6 and PAX6(5a), though the PAX6(ΔPD) isoforms will not be affected. If the PTC is located in exon 5a, especially if caused by a substitution, it is possible that both canonical PAX6 and PAX6(ΔPD) isoforms will not be affected. Mutations that are located in the coding sequence of the linker domain upstream of the translational start sites may produce truncated canonical and PAX6(5a) proteins composed only of an intact paired domain, with the ability to present selective dominant negative effects, and the PAX6(ΔPD) isoforms will remain unaffected.

Mutations at or after the translational start sites in the linker domain may lead to arguably more severe dominant negative proteins, especially if the PTC is found after the homeodomain sequence. These dominant negative proteins would have intact DNA binding domains in all three isoforms, competing with wild-type PAX6 but failing to work as a transcriptional effector because of the loss of function of the transactivation domain. Overall, transcripts with PTC-causing mutations are capable of producing very different PAX6 protein products. However, these transcripts are putatively subject to RNA surveillance mechanisms that would lead to haploinsufficiency.

The nonsense-mediated mRNA decay (NMD) pathway is the primary RNA-surveillance mechanism likely acting on PAX6 transcripts harboring a PTC. NMD identifies these aberrant transcripts and degrades them before they can be translated into proteins that would have dominant negative effects, gain of function effects or otherwise inefficiently use cellular resources and contribute to noise in the interactome (Chang, Imam, & Wilkinson, 2007; Hentze & Kulozik, 1999). NMD acts on transcripts during the primary round of translation (when mRNA has a CBP20/CBP80 5' cap), and requires a number of cellular factors to identify and degrade mutant transcripts (Chang et al., 2007; Ishigaki, Li, Serin, & Maquat, 2001). This process acts by recognizing PTCs upstream of the final exon junction complex (EJC) during translation, forms a complex that dissociates the ribosomal subunits and targets the mutant transcript for degradation by the RISC complex (Chang et al., 2007). There is also recent evidence that NMD can act on eIF4E-bound mRNA transcripts after the primary round of translation, though this is still under investigation. It has become increasingly evident that consideration of the nonsense-mediated decay RNA surveillance pathway is essential in our understanding of genetically-mediated disease, and has been extended to inform targeted genetic

therapies for patients with PTCs in disease causing genes (Bhuvanagiri, Schlitter, Hentze, & Kulozik, 2010; Holbrook, Neu-Yilik, Hentze, & Kulozik, 2004). Until recently, the pathway has been conventionally viewed as a ubiquitous phenomenon, occurring indiscriminately across time and tissue type. However, studies demonstrating spatial and temporal variability of NMD have emerged, implicating this pathway as a key factor in decoding the molecular and phenotypic manifestation of genetically mediated human disease.

The spatial, temporal, and individual variance in NMD function and efficiency is currently a topic of great interest, but many aspects of NMD control and variation have yet to be investigated. Recent work has implicated miR-128 in the down-regulation of NMD activity by binding and degradation of UPF1 and MNL51 transcripts. The UPF1 gene encodes an RNA helicase that is required for NMD and MLN51 encodes an exon junction complex protein that promotes NMD factor binding in mammals (Chang et al., 2007; Rehwinkel, Raes, & Izaurralde, 2006). Both UPF1 and MNL51 have seed sequence matches to miR-128 in the 3' UTR, and the binding of miR-128 as a direct repressor in both cases has been validated in *in vitro* experiments (Bruno et al., 2011). These experiments demonstrated not only the down-regulation of UPF1 transcript survival in the presence of developmentally relevant levels of miR-128, but also that of NMD activity as a consequence by showing an increase in mutant target transcript survival under these conditions. These findings are of particular interest in neurodevelopmental disorders such as aniridia, as miR-128 expression is significantly up-regulated in the nervous system during early development with a high degree of conservation across mammals (de Lima Morais & Harrison, 2010). Further, the fluctuations in this pathway are a prime suspect in the investigation of preliminary evidence showing the presence of gene products that are known targets of NMD.

A secondary RNA surveillance pathway known as staufen-mediated mRNA decay (SMD) is mechanistically similar to NMD (Park & Maquat). SMD may increase the efficiency of degradation of mutation transcripts in the cell but, because SMD is also dependent on UPF1, it is likely subject to the same spatiotemporal variance directed by miR-128 expression. In the case of PAX6, if NMD and SMD activity is suppressed during specific stages of neural development, then truncated protein products would be present during this time course. It follows that the fluctuations of these transcriptional interactions could ultimately have large phenotypic implications, which demands a closer inspection of spatiotemporal PAX6 mutant protein expression. Just as the discovery of this pathway changed the way the scientific community understands genetically mediated diseases such as aniridia, future work elucidating the nuances and dynamics of NMD and SMD will substantively refine the working model of transcriptional-level mechanisms in genetic disease as well as the implications of these mechanisms for disease phenotypes.

Another surveillance mechanism that may be acting on PAX6 transcripts harboring a PTC is nonsense-associated altered splicing (NAS). NAS is a mechanism that up-regulates alternatively spliced transcripts that skip PTCs . For example, if a PTC-causing mutation is found in exon 5a of PAX6, NAS would putatively up-regulate canonical PAX6 transcripts relative to PAX6(5a) transcripts during mRNA processing. Alternatively, if a mutation is found at the 3' end of an exon between two possible exonic splice recognition sequences, the exon may be retained but recruitment of alternative splice sites could remove the PTC sequence. In the case of a frameshift mutation leading to a PTC in which the PTC is spliced out by NAS but a shift in the ORF remains, this could exacerbate the consequences of the mutation because the mutant transcript would not be subject to NMD but could still lead to a non-functional or

dominant negative protein. NAS can be thought of as a positive cellular response to correcting genomic PTC errors in the ORF by rescuing transcripts towards production of functional proteins, while NMD and SMD can be thought of as negative responses to this phenomenon by preventing the translation of truncated proteins. The function of this mechanism in the context of PAX6 mutations depends largely on the location of the mutation and its neighboring sequences.

Mutations are often classified solely by their nature or the change that they effect in the ORF of a gene. To fully understand the molecular mechanisms governing individual-specific mutations, gene products, and their effects at the cellular level, we must also consider the location of the mutation relative to differential isoform expression, cryptic splice sites, regulatory element binding, and the susceptibility of the transcripts to RNA surveillance mechanisms in time and space. These factors could act in a combinatorial manner to alter PAX6 transcription and translation in a way that is specific to individual mutations, and should be considered in evaluating the higher-order effects of mutations on cellular dynamics, systems and clinical symptoms of aniridia, especially in the brain.

5.1.2 A circuit and systems perspective

The PAX6 protein is an integral upstream regulator in both cell autonomous and non-cell autonomous processes that contribute to neural progenitor fate decisions, corticogenesis, and the formation and maintenance of neural circuits. As such, mutations that reduce PAX6 function have far-reaching effects on cortical organization, local neuronal connections and establishment of cognitive and sensory processing circuits. Phenotypic evidence suggests that some of these circuits are more severely affected by heterozygous loss of function mutations of PAX6, while haploinsufficient levels of the protein or adaptive mechanisms may be sufficient for the functionality of others.

In aniridia, deficits in olfaction, visual and auditory sensory processing, circadian regulation, resting state connectivity, and working memory have been reported. These abnormalities are accompanied by neuroanatomical changes, namely reductions in interhemispheric commissures and the pineal gland. Additional anatomical abnormalities have been reported in grey matter, which may also be contributing to sensory processing abnormalities. Examining anatomical deficits has provided substantive evidence for the neurophysiological origins in some, but not all, sensory abnormalities. For example, reductions or accelerated deterioration of the pineal gland has been linked with circadian dysregulation in aniridia (Hanish, Butman, Thomas, Yao, & Han, 2015; Mitchell et al., 2003). Prior to our current work, auditory processing deficits have been attributed to reductions in the anterior commissure and corpus callosum, which do not fully explain the functional impairments observed in patients. In Chapter 4, we employed electroencephalography (EEG) to query neural signals associated with auditory processing. We found that examining anatomical structure alone is not sufficient to understand complex circuit-level changes, and that both subcortical, early cortical, and late cortical integration processes are differentially altered in persons with aniridia. Further, in Chapter 3, we found that heterozygous *Sey*^{Neu} mice show volumetric differences in auditory associated cortices as measured by high resolution MRI. Together, our findings suggest that a combination of neuroimaging approaches, in both humans and model organisms, may be the key to elucidating the structural and functional basis of circuit and behavioral-level phenotypes caused by PAX6 mutations. Furthermore, this approach would likely be applicable to various genetically-mediated disorders that present with complex sensory and/or cognitive phenotypes, as neuroimaging provides a bridge between human and model organism study for direct comparison.

5.1.3 An integrated perspective

The direct mechanistic link between PAX6 deficiency and disruptions in sensory and cognitive processes, as opposed to anatomical correlates, has not been extensively studied. To fully understand the causal sequence of events between molecular mutations and circuit/behavioral consequences, future experiments should test hypotheses based on inference from both molecular and systems-level studies. In auditory processing, for instance, the subcortical and cortical disruptions proposed in Chapter 4 could be directly measured using electrophysiology in mouse models. Further, manipulating the molecular nature of PAX6 mutations or the processes that govern expression of PAX6 products in these experiments would more specifically identify the biological disruptions that are sufficient to cause neurophysiological deficits in cellular networks. Additionally, molecular characterization of cell types and synaptic connectivity could be used to better understand the link between molecular and system changes in aniridia, as well as the establishment and maintenance of the mammalian auditory processing system in general.

5.2 Evaluating and developing new treatments for aniridia

5.2.1 A molecular and cellular perspective

Treatments of genetically-mediated diseases through the manipulation of molecular mechanisms are rapidly gaining momentum. Pharmaceuticals that address genetic mutations at the transcriptional and translational level have reached the clinic, and new techniques in genome editing are being refined towards personalized medicine. In order to consider these approaches for treatment of aniridia, a thorough understanding of individual differences in mutant gene products and how these products are handled in the cell is imperative. The characteristics of mutations and subjectivity of their mRNA and protein to cellular processes discussed in section

5.1.1 are essential for selecting individuals as candidates for new therapies, optimizing treatment plans and predicting treatment outcomes.

A large percentage of PAX6 mutations causal for aniridia are PTCs. If transcripts harboring these mutations are translated or if they are degraded by the NMD or SMD pathways, truncated proteins with possible dominant negative effects or PAX6 haploinsufficiency would result, respectively. Recently, a small molecule pharmaceutical known as ataluren has shown promising results in preventing both of the aforementioned consequences of PTC-containing transcripts. Ataluren (formerly PTC124) acts by promoting ribosomal read-through of PTCs, treating early stop codons as missense mutations, and enables translation of full-length proteins which putatively retain function (Du et al., 2008; Welch et al., 2007; Yu et al., 2014). This drug has shown measurable results in duchenne's muscular dystrophy and cystic fibrosis cases caused by PTC-causing mutations (Bushby et al., 2014; Kerem et al., 2014; Sermet-Gaudelus et al., 2010; Wilschanski et al., 2011), and demonstrated partial or full rescue of ocular phenotypes in heterozygous *Sey* mice when administered systemically and topically after birth (Gregory-Evans et al., 2014). In section 2.4, individuals with mutations that would be ideal candidates for clinical application of ataluren are discussed based on *in vitro* testing of this drug. However, the codons examined for optimal efficacy of ataluren were not done so in the context of the START drug formulation that is being applied in *in vivo* testing of animal models and proposed for clinical cases of aniridia (Gregory-Evans et al., 2014; Yu et al., 2014). It is possible that this formulation or mutations of the PAX6 gene specifically will demonstrate different responses than previous studies on PTC codon sequence. In addition, prenatal administration and long-term exposure effects of ataluren have yet to be investigated, and caution should be observed in these contexts as clinical use of the drug proceeds.

Ataluren does not act in a gene-specific manner, so it likely ubiquitously promotes ribosomal read-through of transcripts with PTCs that are normally degraded by NMD. In addition to disease-causing gene transcripts, these could include transcripts with copy errors introduced during translation, those from mutations in genes that would otherwise not cause detrimental outcomes in the system because of NMD, NAS, or SMD mediation, or those that are regulated by RNA surveillance such as NMD in a precise spatiotemporal way to support global processes such as development. For example, ataluren administration during neurodevelopment is appealing because this is when persons with PTC-causing mutations in PAX6 are likely expressing truncated and possibly dominant negative proteins. Meanwhile, the NMD factor UPF1 promotes undifferentiated cell states in part through NMD regulation during development (Lou et al., 2014). The up-regulation of miR-128 and down-regulation of UPF1 and NMD have been shown to trigger neural differentiation (Bruno et al., 2011; Lou et al., 2014). If ataluren interferes with these processes during development it could lead to early or inappropriate neural differentiation and have tremendous effects on neurodevelopment independent of PAX6 protein correction. The inhibition of NMD has also been shown to activate autophagy, while the hyper-activation of NMD suppresses the autophagy response to various cellular stresses (Wengrod et al., 2013). If ataluren is acting on NMD targets in this pathway, this could cause inappropriate regulation of the autophagy response and have dire consequences. In another case, errors during transcription of non-mutant genes periodically introduce PTCs into the ORF, usually corrected by NAS or NMD (Karam, Wengrod, Gardner, & Wilkinson, 2013; Rehwinkel et al., 2006; Wang, Chang, Hamilton, & Wilkinson, 2002). Circumventing the cellular mechanisms which correct for these occurrences before erroneous proteins are introduced into the system could have

a cumulatively detrimental effect on cellular processes in an unpredictable way, especially with chronic drug treatment.

Another consideration in the application of ataluren to PAX6-mediated aniridia is the autoregulatory function of PAX6. PAX6 has both positive and negative autoregulatory activity of its own expression in different contexts (Aota et al., 2003; Pinson, Simpson, Mason, & Price, 2006). In the case of positive autoregulation, ataluren would putatively return decreased expression levels back to normal by increasing the number of PAX6 proteins present in the cell. In negative regulation, this may be detrimental because without the drug the cell may continue to express the wild-type allele of PAX6 until it reaches a detectable threshold. This aspect of PAX6 function also presents a case in which the location of the mutation being skipped by ataluren is of critical importance: two specific subdomains of the paired domain are involved in precise control of PAX6 autoregulation (Yamaguchi, Sawada, Yamada, Handa, & Azuma, 1997). If one of these domains is conformationally or biochemically affected by a synthetic "missense" codon introduced by ataluren facilitated translation, this process could be considerably altered.

Despite the necessity of further investigation of off-target effects of ataluren, this drug shows potential for a major advance in the treatment of aniridia. However, the use of ataluren is currently restricted to cases of aniridia in which the causal mutation contains a PTC caused by a single nucleotide substitution. For instance, ataluren treatment of a patient with a frameshifting mutation that leads to a PTC would likely cause more harm than good because the protein introduced would not likely retain PAX6 function and could have dominant negative effects. Genome editing to correct any known mutation of PAX6 is an option for the future, though the application of TALEN or CRISPR-Cas9 is not yet optimized for human germ line use (Bedell et

al., 2012; Ding et al., 2013; Ran et al., 2013; Veres et al., 2014). Other pharmaceuticals that target alternative mechanisms governing PAX6 expression, such as NAS, should be explored.

5.2.2 A circuit and systems perspective

Correcting genetically-mediated disease outcomes from the "ground up" by addressing molecular changes is ideal, but is not always plausible. Understanding sensory and cognitive processing abnormalities as they manifest in aniridia provides new avenues for intervention towards enhancing quality of life for patients. Living with visual deficits is already a focus in the clinic for the treatment of aniridia patients, but a similar approach can be taken when addressing auditory processing, working memory, and learning related deficits that often accompany the disease.

Auditory processing abnormalities have been indicated in a considerable percentage of patients with aniridia. These abnormalities were demonstrated by poor performance on central auditory tests including dichotic speech tests, frequency and duration pattern tests, and gaps-in-noise tests (D. E. Bamiou et al., 2004, 2007). These are comparable to characteristics of central auditory processing disorder (CAPD), so it follows that treatments that are successful in CAPD may also be useful if incorporated in treatment plans for aniridia (Pelchar, 2011). A broad approach for intervention has demonstrated success in CAPD, combining what are deemed as "bottom-up" and "top-down" comprehensive training ((ASHA), 2005). Successful interventions include acoustic signal enhancement, auditory training, cognitive, and language strategies (Baran, 2002; Chermak & Musiek, 2002; Kilgard & Merzenich, 1998; Musiek, Shinn, & Hare, 2002). Future work should evaluate these interventions as a possible addition to aniridia treatment.

Working memory deficits have also been associated with aniridia (D.-E. Bamiou et al., 2007; Thompson et al., 2004), and the association between working memory and other cognitive processes such as attention and learning has been extensively studied (Kane & Engle, 2003; Kane et al., 2004; Kirschner, 2002; Unsworth, Spillers, & Brewer, 2010). The assessment of individual differences in working memory and attentional processes in the context of optimizing learning, especially in the classroom, is a topic of great current interest. Understanding variation in working memory and learning in children with aniridia, in conjunction with identification of auditory processing deficits, may help provide insights towards tailoring classroom instruction for these individuals (Kirschner, 2002). Further research should consider working memory and learning assessment in conjunction with auditory processing intervention in this population.

5.2.3 An integrated perspective

The severity and progression of aniridia is commonly evaluated based on the predominant ocular symptoms of the disorder. However, evidence is rapidly emerging that implicates extraocular symptoms as central characteristics of aniridia. It is imperative that, as we refine our understanding of the disorder, we also reexamine individual differences and possible genotype-phenotype correlations. Obtaining comprehensive diagnostics spanning neural, cognitive, and metabolic phenotypes in addition to ocular measures and genotypes will provide (i) a better indication of required individual treatment plans, (ii) a more thorough context for disease progression and variation, and (iii) additional means by which to evaluate patient response to genetic-based treatments. The advancement of treatment for aniridia requires that we consider the entire complex of associated symptoms, as well as the molecular context surrounding individual genetic mutations.

5.3 A potential role for PAX6 in neural processing and other disorders

Anatomical and functional consequences of PAX6 mutations in model organisms and humans suggest that PAX6 plays an important role in the precise establishment, organization, and maintenance of specific neural circuits. In addition to improving our understanding of known PAX6-mediated disorders, studying the models available and investigating the nuances of cognitive and sensory processing in humans with PAX6 mutations presents an opportunity to explore these circuits from both a molecular and systems approach. Where loss-of-function mutations of PAX6 cause disruptions in neural circuits, they may also open a doorway to explore the genomic networks and cellular mechanisms that are responsible for complex cognitive processes. This could lead to a better understanding of individual variation in cognition and sensory processing, as well as identify molecular candidate networks for associated disorders.

Various mutations in model organisms and reports by aniridia patients implicate PAX6 in auditory processing, working memory, olfactory function, sensorimotor deficits, and neuroanatomical abnormalities. In the case of sensorimotor function, knockout of PAX6 causes deficits in *Drosophila* but heterozygous mutations are not sufficient to elicit this phenotype in mice or humans. Furthermore, changes in cortical grey matter in sensorimotor associated areas were detected in Sey^{Neu} mice as discussed in Chapter 3. It is therefore likely that PAX6 is involved in the cellular interactome that directs the organization of this circuitry, but its role is not as critical as it is in establishment of visual or auditory circuitry.

As previously described, missense mutations of PAX6 have been associated with autism spectrum disorders and isolated ocular abnormalities. It is possible that small molecular changes in PAX6 which alter the function of protein domains in discrete ways but do not lead to global reduction in PAX6 function could be associated with other phenotypes of aniridia independently.

For example, central auditory processing disorder, working memory-associated cognitive impairment, or olfactory dysfunction may be associated with these types of mutations. Alternatively, the direct interactions and downstream targets of PAX6 in the establishment of these neural circuits could identify candidate genes in these disorders or other associated disorders such as autism. Future work should investigate these molecular hypotheses in other disorders and explore PAX6-related network variation in the context of individual differences in cognitive and sensory processes.

5.4 Conclusion

The studies presented here provide insights into the variability of molecular mutations in persons with aniridia, the conserved and human-specific neuroanatomical abnormalities associated with PAX6 mutations, and the first functional assessment of auditory processing in humans with this disorder. Collectively, this work emphasizes the utility of employing multiple approaches, spanning molecular, model organism, and human investigation, in understanding complex genetically-mediated diseases affecting the brain. Because of the prominent role of PAX6 in neurodevelopment and the specific deficits associated with mutations of this gene in humans, this line of investigation provides a unique opportunity to understand not only the link between PAX6 and brain-related phenotypes, but also a framework for investigating the relationship between molecular and cellular changes and neurophysiological consequences of genetically-mediated neurodevelopmental processes.

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