Functional characterization of *Dlx* enhancers in the developing mouse

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Abstract

The *Distal-less homeobox* (*Dlx)* genes encode homeodomain transcription factors found in all animals of the phylum Chordata. These genes are involved in early vertebrate development of limbs, sensory organs, branchial arches and the forebrain (telencephalon and diencephalon). The mouse and human genomes each have six *Dlx* genes organized into convergently transcribed bigene clusters (*Dlx1/2, Dlx3/4* and *Dlx5/6*). In the forebrain, *Dlx1/2* and *Dlx5/6* genes play essential roles in GABAergic neuron proliferation, migration and survival. Each bigene cluster includes a short intergenic region (~3.5-16kb) harboring *cis*-regulatory elements (CREs) that control expression of the *Dlx* genes. The *Dlx1/2* intergenic region harbors the I12b/I12a CREs, while *Dlx5/6* includes I56i/I56ii. In determining the regulatory roles of the CREs on *Dlx* activity and forebrain development, I have characterized the phenotypic changes that occur in mice that have an I56i enhancer deletion. I have also characterized mice with double deletions of I56i and I12b as well as mice that harbored an I12b deletion and have a SNP in the I56i enhancer (vI56i). Mutant mice with a single targeted deletion of I56i are viable, fertile and do not show obvious developmental defects. These mice have significant decreases in *Dlx5/6*, *Gad1/Gad2* and *Evf-2* expression in the forebrain and have defects related to GABAergic neuron development. The ΔI56i mutants demonstrate a behavioral phenotype related to anxiety and learning deficits. Mice that lack the I12b enhancer and have the vI56i do not show morphological abnormalities but have severely disrupted *Dlx* expression. When mice are homozygous for the I56i and I12b enhancer deletion, they do not survive past post natal day 5 and exhibit a dwarfed body size. These mice look weak and seem to have limited motor ability. In characterizing mice with targeted deletions of highly conserved *Dlx* enhancers, we will have a better understanding of forebrain development.

Résumé

Les gènes á homéoboîte Distal-less (*Dlx*) codent pour des facteurs de transcription á homéodomaine trouvés chez tous les animaux de l'embranchement Chordata. Ces gènes sont impliqués dans le développement précoce des membres, des organes sensoriels, des arcs branchiaux et du cerveau antérieur (télencéphale et diencéphale). Le génome de l'homme et de la souris possèdent chacun six gènes *Dlx* dont l'organisation résulte en la convergence de la transcription de deux gènes du cluster (*Dlx1/2, Dlx3/4* et *Dlx5/6*). Dans le cerveau antérieur, les gènes *Dlx1/2* et *Dlx5/6* jouent un rôle essentiel dans la prolifération, la migration et la survie des neurones GABAergiques. Chaque bigène inclut une courte région intergénique (~3,5-16kb) abritant des éléments cis-régulateurs (CREs) qui contrôlent l'expression des gènes *Dlx*. La région intergénique *Dlx1/2* abrite les éléments cis-régulateurs I12b et I12a tandis que la région *Dlx5/6* comprend les éléments I56i et I56ii. Afin de déterminer les rôles régulateurs de CREs sur l'expression des *Dlx* et sur le développement du cerveau antérieur, nous avons caractérisé les variations phénotypiques qui se produisent chez les souris ayant subis une ablation ciblée de I56i. J'ai aussi évalué le phénotype de souris qui n'ont ni l'activateur I56i, ni l'activateur I12b. Les souris mutantes possédant une délétion de I56i sont viables, fertiles et ne démontrent aucune anomalie évidente du développement. Ces souris montrent des diminutions significatives de l'expression des gènes *Dlx5/6*, *Gad1/Gad2* et *Evf-2* dans le cerveau antèrieur et montrent des anomalies associès au développment des neurones GABAergiques. Les mutants ΔI56i montrent un phénotype comportemental unique et semblent être anxieux et semblent posséder des troubles d'apprentissage. Lorsque les souris sont homozygotes pour la délétion des amplificateurs I56i et I12b, leur durée de vie n'excède pas la journée post-natale 5 et elles présentent une taille réduite. Ces souris semblent faibles et avoir des capacités motrices limitées. La

caractérisation de souris ayant des ablations ciblées des amplificateurs *Dlx* hautement conservés nous permettra de mieux comprendre les mécanismes qui contrôlent le développment précoce du cerveau antérieur.

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Abbreviations and Acronyms

ASD: autism spectrum disorder

BAC: bacterial artificial chromosome

bp: base pairs

CGE: caudal ganglionic eminence

ChIP: chromatin immunoprecipitation

Dll: *Distal-less*

Dlx: *distal-less homeobox*

E11.5: embryonic development day 11.5 after conception

E13.5: embryonic development day 13.5 after conception

Evf-2: embryonic ventral forebrain-2

FH: fetal hemoglobin

GABA: γ-aminobutyric acid

Gad: glutamic acid decarboxylase

GE: ganglionic Eminence

LGE: lateral ganglionic eminence

Mash1: mammalian achaete scute homolog-1

mI56i: mouse I56i enhancer

MGE: medial ganglionic eminence

MZ: mantle zone

ncRNA: non-coding ribonucleic acid

P0: postnatal day 0 (birth)

PBS: phosphate buffered saline

PFA: paraformaldehyde

qRT- PCR: Quantitative Real-Time PCR

RNA: ribonucleic acid

RT: room temperature

SNP: single nucleotide polymorphism

SVZ: subventricular zone

trucRNA: transcription-regulating ultraconserved non-coding ribonucleic acid

URE1: upstream regulatory element 1

URE2: upstream regulatory element 2

vI56i: variant form (with the SNP) of the I56i enhancer

VZ: ventricular zone

List of Figures and Tables

Statement of Contributions

Crystal Esau executed all experiments presented in this thesis. However, Tanya Plaoude a second year undergraduate student sectioned some of the mouse forebrain tissue and helped genotype the various mouse lines used in this thesis. The E14.5 *in situ* hybridizations and immunohistochemistry experiments were performed by Crystal Esau and Tanya Plaoude together.

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Thank you to my undergraduate student Tanya Plaoude who showed ambition and determination towards my project from day one. It was a pleasure teaching and working with a student with such a positive attitude and natural talent. I am sincerely grateful for your hard work and friendship.

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1 Introduction

1.1 Nervous System Development in Vertebrates

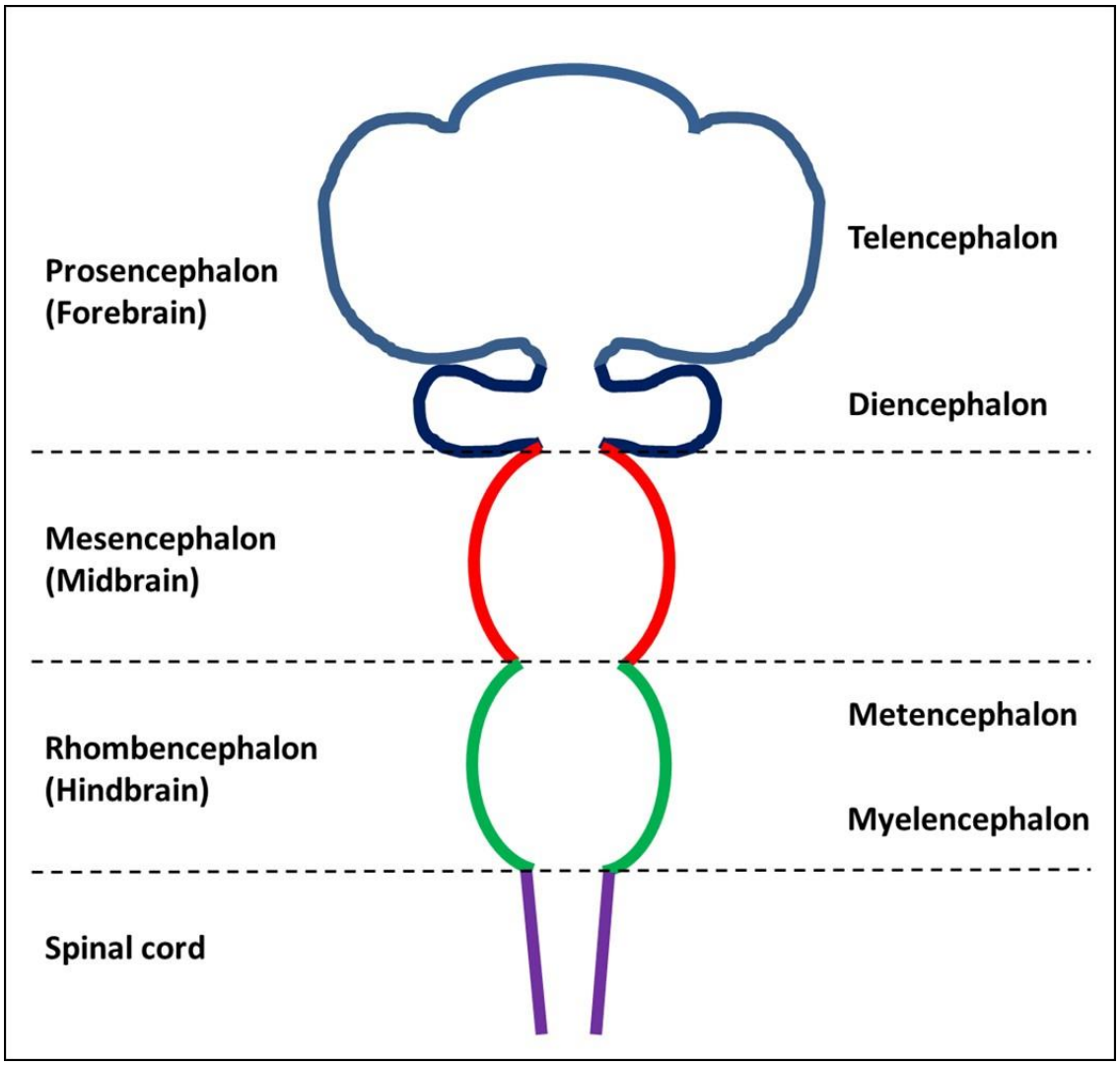
During early embryonic development the onset of gastrulation is accompanied by the induction of the nervous system. During gastrulation, the blastula which is a hollow sphere of cells transforms into a gastrula composed of three germ-layers; the ectoderm, mesoderm and endoderm. Through a multiplex of signals, the nervous system arises from the segregation of the ectoderm into a neuronal portion called neuroectoderm, which is the most dorsal region of this peripheral germ layer. Ectodermal tissue overlaying the notochord begins to thicken and roll upwards on the dorsal midline, forming the neural plate. The neural folds begin to fuse and join over the midline, eventually forming a hollow cerebrospinal fluid-filled neural tube. The caudal portion of the neural tube remains as a tubular structure forming the spinal cord while the anterior portion undergoes numerous constrictions, swellings and flexures that pinch off into three major structures of the brain along the anterior-posterior axis that will eventually subdivide into five secondary structures (Nicholls et al. 2012). The mammalian brain is first divided into the hindbrain (rhombencephalon) from the combined forebrain (prosencephalon) and midbrain (mesencephalon) until the next set of divisions that form the prosencephalon, mesencephalon and rhombencephalon. Lastly, the prosencephalon is split into the telencephalon and diencephalon and the rhombencephalon is cleaved into the metencephalon and myelencephalon (Fig. 1.1) (Wolpert and Tickle 2011).

In this study, the developing forebrain is of interest, particularly the telencephalic domain which encompasses the cerebral cortex (hippocampus and neocortex), basal ganglia and olfactory bulbs (Zaki et al. 2003). The function of the telencephalon includes sensory processing, communication, learning, voluntary movement and memory (Roth and Wullimann 2001). The telencephalon is derived from the anterior neural plate and it's early

development is specified by numerous signaling molecules within the telencephalon itself and surrounding tissue. Once the anterior neuropore closes, morphogenetic changes occur through the migration of telencephalic cells from an anterior position to a more ventral location, resulting in the regionalization of the telencephalon (Fishell 1997). A distinct set of regional markers including the *Dlx* genes are expressed following neuropore closure, differentiating discrete proliferative zones in the pallial (dorsal) and striatal (ventral) telencephalon. These proliferative zones give rise to five structures that make up the telencephalon; cortex, striatum, pallidum, septum and limbic system which are responsible for the highest level of neural function in vertebrates (Fishell 1997).

The ganglionic eminences (GE) are found in the ventral ventricular zone of the telencephalon and are present during the embryonic and fetal stages of neural development, guiding tangential cell and axon migration. These regions of the brain guide axons from the thalamus into the developing cortex. In this study, the ganglionic eminences are significant in that they play a role in building up a GABAergic cortical interneuron population and guide these neurons to their respective location in the brain. The subventricular zone (SVZ) is located next to the ventricles and GE of the forebrain. It serves as a site for neural stem cells and self-renewing neurons, promoting cellular proliferation. The *Dlx* genes are expressed in the ventricular zone (VZ) and SVZ of the GE's in the telencephalon, with specific patterns for their individual paralogs. Disruptions in these genes may cause a dysfunction in GABAergic neuron proliferation and differentiation, leading to neurological disorders (Fishell 1997).

Figure 1.1: Schematic representation of the developing mammalian brain. The mammalian embryonic brain differentiates into the forebrain (telencephalon and diencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon) (Courtesy of Jacob Pollack).


Prosencephalon
(Forebrain)
Telencephalon
Diencephalon
Mesencephalon
(Midbrain)
Rhombencephalon
(Hindbrain)
Metencephalon
Myelencephalon
Spinal cord

1.2 Mammalian Neurogenesis

In mammals, the nervous system functions as a communication system made of a large network of electrically excitable neuronal cells called neurons. The brain contains approximately 100 billion neurons of various size and functions along with non-neuronal cells such as oligodendrocytes and astrocytes, collectively known as glia that act as support cells. Neurons are specialized cells that form electrical and chemical connections between target cells and with one another, transmitting information to the brain (Wolpert and Tickle 2011). The site of a neuronal connection is known as a synapse. Neurons receive input signals through their dendrites, highly specialized structures that receive stimulation from other neural cells and transmit a new signal (action potential) at the cell body. The electrical signal travels along an axon towards the axon terminal where another synapse is formed with the dendrites of a neighboring neuron or target cell. At the site of the synapse, an electrical signal is converted into a neurotransmitter (chemical signal) and released from a nerve ending to act upon receptors of another target cell, initiating or suppressing a new electrical signal (Wolpert and Tickle 2011).

Neurons and glia arise from progenitor cells in the VZ of the brain which is a proliferative layer of epithelial cells that make up the lumen of the neural tube (Fig 1.2 A). Neural progenitors are multipotent neural stem cells that give rise to both neurons and supporting glia. Through mitotic cell division, neural stem cells will differentiate into either neural precursors or additional stem cells. Neuroblasts, which are differentiated from neural stem cells and are committed to a neuronal fate, migrate from within the neural tube towards the outer surface. The migration pattern of neurons is related to the time they are born and those who are born during early stages of development migrate close to their birth site, while those born later must migrate past the older neurons to more superficial layers of the brain,

building up neural tissue in concentric layers (Wolpert and Tickle 2011). Two major classes of neurons make up the developed mammalian brain, excitatory projection neurons that use glutamate as their chief neurotransmitter and inhibitory interneurons that use γ-aminobutyric acid as a neurotransmitter (Benes and Berretta 2001). Neurons migrate to their final destination either radially or tangentially. Radial migration is the primary mode of migration for developing neurons and is critical to the proper morphogenesis of the cerebral cortex (Fig. 1.2 B). This mode of travel is represented by 90% of migrating neurons and includes somal translocation and radial glia which functions as a platform for migrating cells (Marin and Rubenstein 2001). As cells migrate along glial tracks passing their progenitor cells, concentric layers are formed in an inside-out manner. Tangential migration includes those migrating neurons from the VZ that will eventually give rise to GABAergic local circuit neurons (Fig. 1.2 B). These neurons follow distinct tangential trajectories, migrating to the neocortex, striatum and olfactory bulb (Marin and Rubenstein 2001). Disruptions in the establishment of a functioning neuronal network are associated with abnormal behavior and neurological disease such as anxiety, Rett syndrome and autism.

1.3 GABAergic Neurons

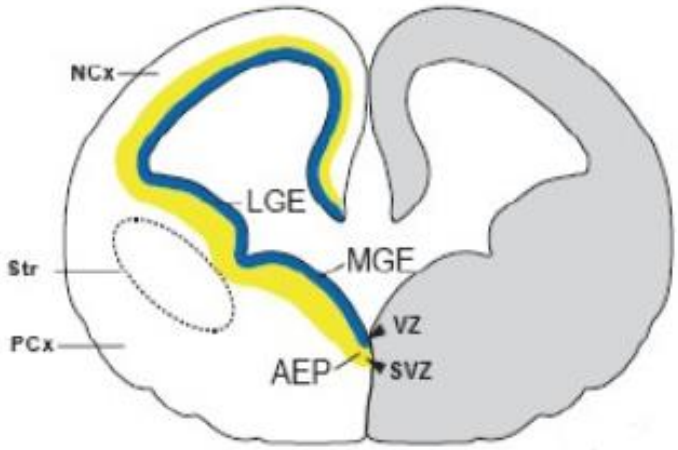
GABAergic interneurons maintain neuronal activity through inhibitory functions using gamma-aminobutyric acid as the chief neurotransmitter and differentiate in overlapping domains with endogenous *Dlx1, 2, 5* and *6* expressions in the telencephalon (Stuhmer et al 2002). GABAergic neurons make up 20% of all neurons found in the cortex and hippocampus and 95% of all neurons in the striatum. The subpallial lateral ganglionic eminence (LGE) is the birthplace for GABAergic interneurons from where they will tangentially migrate towards the olfactory bulb, hippocampus and neocortex (Potter et al.

2009). During the early stages of forebrain development, γ-Aminobutyric acid (GABA) establishes the cortical network through excitatory synapses and then becomes inhibitory once the developing neurons have been excited (Ben-Ari 2002). These neurons can be subdivided into different groups based on their connectivity, morphology, and electrophysiological properties. The first class of GABAergic neurons are basket cells which are the most commonly encountered interneuron and have axon-somatic inhibitory synapses. Chandelier cells, a second group of GABAergic neurons, have axonal branches extending at right angles forming "candles" in a vertical orientation and have axo-axonal inhibitory synapses. Lastly, double bouquet and tuft cells are a class of GABAergic neurons that distribute themselves within narrow columns of the cortical mantle and have axo-dendritic inhibitory and disinhibitory synapses (Benes and Berretta 2001). The various neuronal subtypes are identified by their expression of neurochemical markers such as the calcium binding proteins calbindin, parvalbumin, calretinin, somatostatin, and neuropeptide-Y (Potter 2009).

The inhibitory neurotramsitter GABA is needed to transmit chemical signals from a neuron to a target, initiating a synapse. GABA is synthesized from glutamate through the enzyme glutamic acid decarboxylase (Gad) which has two isoforms, *Gad65/Gad67* which are commonly referred to as *Gad1/Gad2* respectively. Approximately 95% of both Gad isomers are co-localized in all GABA cells of the hippocampus. *Gad* is primarily expressed in axon terminals and in the somata and dendrites of $GABA^{+}$ cells (Benes and Berretta 2001). The principle function of GABA is to control the output of principle excitatory neurons through negative feedback. The GABA neurotransmitter functions by activating chloride conducting receptors which initiate an inhibitory postsynaptic potential in postsynaptic

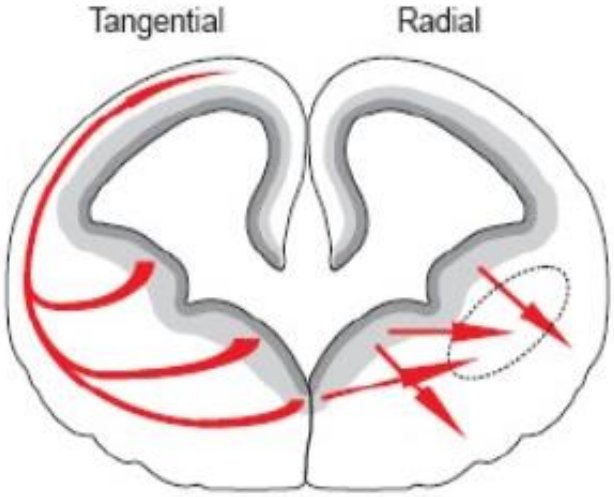
Figure 1.2: Coronal cross sections of a basal telencephalon of an E12.5 mouse embryo. **(A)** Various structures of the telencephalon **(B)** Radial and tangential migration pathways of neurons during development (Courtesy of Cindy Pelletier).

A)



AEP: Anterior entopeduncular area
LGE: Lateral ganglionic eminence
MGE: Medial ganglionic eminence
NCx: Neocortex
PCx: Piriform cortex
Str: Striatum
SVZ: Subventricular zone
VZ: Ventricular zone

B)



neurons. As the chloride conducting receptors become activated, there is a sharp increase in conductance, inhibiting excitatory postsynaptic potentials (Nicholls et al. 2012).

The *Dlx* genes are virtually expressed in all $GABA^+$ cells and aid in the migration and differentiation of GABAergic neurons. In *Dlx1/Dlx2* double mutant mice, there are defects in neuronal migration, maturation and a decrease in number of GABAergic projection and interneurons, demonstrating the importance of DLX1 and DLX2 in the development of GABAergic neurons. *Dlx1/Dlx2* double mutants also have a reduction in *Gad* expression, which is responsible for synthesizing GABA from glutamic acid (Anderson et al. 1997a). *Dlx5* mutants exhibit defects in the differentiation of interneurons found in the olfactory bulb (Anderson et al. 1997b).

1.4 The *Distal-less (Dll) G*ene

Distal-less (Dll) is one of the most primitive genes involved in distal limb development of insects. It encodes a homeodomain transcription factor that is responsible for the formation of larval sense organs and is essential for promoting growth required to elongate appendages with a proximodistal axis. In *Drosophila, Dll* is expressed during early embryogenesis in the developing limbs, antennae, optic lobe of the brain and glial cells of the ventral nerve cord (Kaphingst and Kunes 1994). The invertebrate species studied thus far have only one *Dll* gene, while vertebrates have multiple homologous *Dll* genes called *distal-less homeobox* genes (*Dlx). Drosophila Dll* mutants die as embryos and do not form larval antennae, maxillary sense organs or Keilin's organs (larval legs) (Panganiban 2000). While the role of *Dll* in the nervous system is currently unknown, mutations in vertebrate *Dlx* genes show defects in brain development. Because *Dll* and *Dlx* are both expressed in the nervous system of a variety of invertebrates and vertebrates, including the limbless *Caenorhabditis*

elegans, it has been suggested that the functional use of *Dll* in the nervous system may predate the evolution of limbs (Panganiban 2000).

1.5 Vertebrate *Distal-less Homeobox (Dlx)* Genes

1.5.1 Transcription Factors and *Homeobox* Genes

The expression of developmental genes is under tight control and requires regulatory regions that harbor elements able to read a set of instructions that turn genes on and off in the right cell type at the right time. Transcription factors are regulatory proteins and are in part responsible for activating and repressing transcription into RNA. They function by binding to specific DNA sequences such as a promoter that will either block or promote the recruitment of RNA polymerase to particular genes. A unique feature of transcription factors are that they contain DNA binding domains, allowing them to attach to target sequences close in proximity to the genes they are regulating. One class of transcription factors that are crucial in regulating developmental genes are the *homeobox* genes (Wolpert and Tickle 2011). These genes are distinguished by the presence of a homeobox, which is a 180 nucleotide stretch of DNA that encodes a DNA-binding-domain called the homeodomain. The homeodomain is a structural domain of 60 amino acid helix-turn-helix configuration that interacts directly with DNA (Alberts et al. 2007). All vertebrate *DIx* genes contain a homeobox and are highly modulated through regulatory elements that help ensure the target developmental genes are regulated at the appropriate time and place.

1.5.2 Regulatory Elements

Transcriptional regulation in eukaryotic cells is a complex process that makes use of gene regulatory proteins and elements. Regulatory elements are non-coding and can operate

thousands of nucleotides away from the promoter they are acting on. They are either *cis*, meaning they act on the same molecule of DNA or *trans*, acting on *cis*-regulatory elements from the opposite side of the gene they are controlling (Alberts et al. 2007). The mode of action of these elements may involve the looping out of a DNA sequence between an enhancer and a promoter, allowing activator proteins that are bound to enhancers to come into contact with the proteins bound to a promoter, activating transcription. *Cis*-acting regulatory elements can be grouped into two classes called enhancers and silencers. Enhancers are short segments of DNA located either up or downstream of the genes they are regulating and can be bound with proteins, activating transcription (Alberts et al. 2007). In contrast to enhancers, silencers repress transcription by binding transcription regulation factors or repressors that, for example block RNA polymerase from binding to the promoter region of genes (Alberts et al. 2007).

1.5.3 Genomic Organization of Vertebrate *Dlx* Genes

The *Dlx* genes are found in all Chordate phyla and encode homeodomain transcription factors that are essential in regulating early embryonic development. The *Dlx* genes are essential in the development of limbs, sensory organs, branchial arches and most predominately the development and neurogenesis of the forebrain (Merl et al. 2000). Eight *Dlx* related genes have been identified in vertebrates. Six of these genes are found in human and mouse organized in three bigene clusters that are convergently transcribed towards each other (*Dlx1/Dlx2*, *Dlx3/Dlx4* and *Dlx5/Dlx6*) and localized on chromosome 2, 11 and 7 (Fig. 1.3). Each bigene cluster is linked to a unique *Hox* cluster in humans (Panganiban and. Rubenstein 2002). Compared to their mammalian counterpart, zebrafish have eight *dlx* genes, two (*dlx5* and *dlx8*) of which are not yet identified to be linked or part of a known bigene

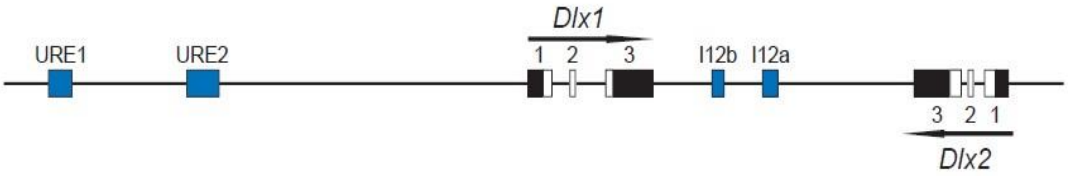
cluster. Each bigene cluster comprises a short intergenic regions (~3.5-16kb) that contains c*i*s-regulatory elements or enhancers that are in part responsible for the regulation of the *Dlx* genes (Ghanem et al. 2003). Each *Dlx* gene has three exons and two introns, possessing a similar organization, with the homeobox region being split between exons 2 and 3 (Liu et al. 1997). The six mammalian *Dlx* genes are organized into two subfamilies based on sequence similarities in the homeodomain of the proteins they encode. The first group includes *Dlx1, Dlx6* and *Dlx4* and the second *Dlx2, Dlx3* and *Dlx5* (Stock et al. 1996).

1.5.4 Evolution of the *Dlx* Genes

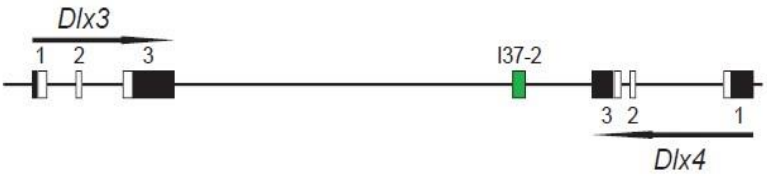
In mammals, the six *Dlx* genes are arranged in three pairs of genes that are convergently transcribed towards one another and are physically linked to a specific *Hox* cluster. The current hypothesis of how these genes evolved in such an organization is thought to have been through tandem duplication events followed by cluster duplications (Fig. 1.4) (Zerucha and Ekker 2000). The evidence for tandem duplication events along a chromosome stems from the mammalian *Dlx* bigene clusters each harboring a member from both of the major *Dlx* groups, which are classified based on sequence similarities of the homeodomains each gene encodes. The first *Dlx* group includes *Dlx1/Dlx4/Dlx6*, while the second includes *Dlx2/Dlx3/Dlx5*. Following the tandem duplication event, it can be assumed that these genes underwent chromosomal duplication and gave rise to multiple linked pairs of *Dlx* genes with the same relative orientation. The *Dlx* cluster duplication event most likely occurred during the time the *Hox* clusters were undergoing genomic duplication. This can be assumed because of the linkage between the *Dlx* and the *Hox* genes coupled with the evidence for their duplication of large chromosomal regions. Through studying invertebrates and

Figure 1.3: Genomic organization of the vertebrate *Dlx* genes. Exons of the *Dlx* genes are numbered with white boxes representing non-coding regions and white boxes representing coding regions. *Dlx* intergenic *cis*-regulatory elements are shown in blue (*Dlx1/Dlx2*), green (*Dlx3/Dlx4*) and red (*Dlx5/Dlx6*) (Courtesy of Dr. Luc Poitras).

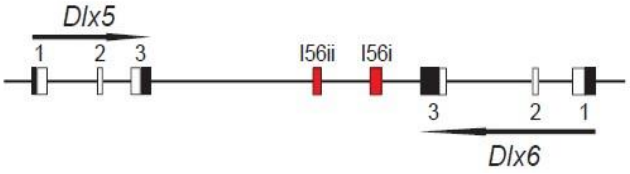
Dlx1/Dlx2



Dlx3/Dlx4



Dlx5/Dlx6



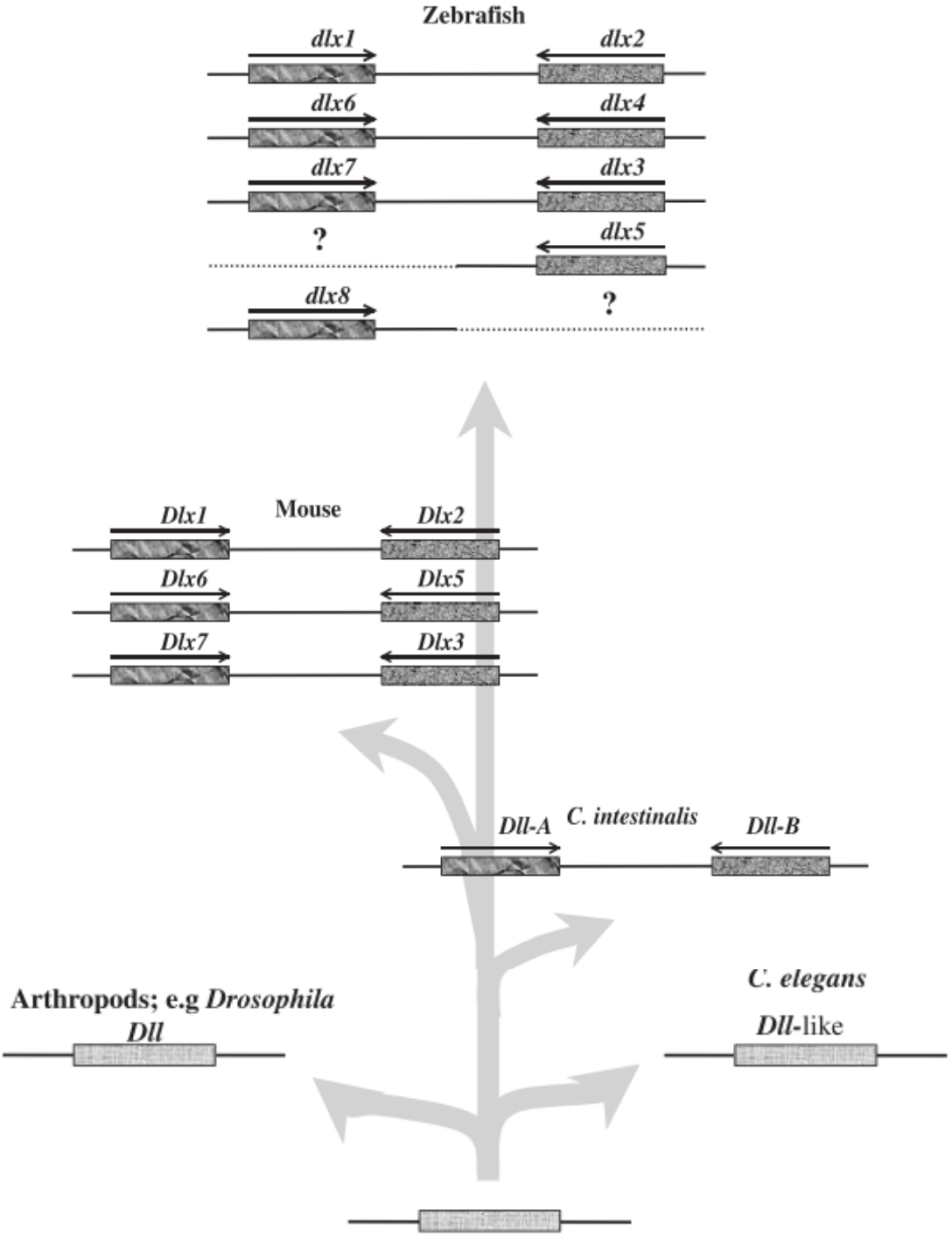
protochordates such as C. *elegans,* which harbors a *Dll* orthologue called *ceh23,* it is possible to provide an estimated time for the initial tandem duplication of the *Dlx* genes (Zerucha and Ekker 2000). Through genomic sequencing in *C. elegans,* a gene was found that had 74% sequence similarity within the homeodomain compared to the *Dll* gene and is linked to a HOM/Hox cluster located on the same side that harbors the most posteriorly expressed gene, similar to that of *ceh-23*. Because there are no other potential *Dlx* homologues within the HOM/Hox cluster in *C. elegans* and only one single *Dlx* locus, it is strongly suggested that *Dlx* duplication events occurred after the divergence of nematodes and eucoelomates (Stock et al. 1996). The identification of a single *Dlx* gene pair in *C. intestinalis* suggests that a tandem gene duplication event may have occurred very early in the evolution of chordates (Zerucha and Ekker 2000).

1.6 Expression of the *Dlx* Genes

1.6.1 Forebrain Expression

In the central nervous system of mammals, four of the six *Dlx* genes (*Dlx1*, *Dlx2*, *Dlx5,* and *Dlx6*) are expressed in the forebrain, particularly in the majority of neural progenitor cells of the ventral telencephalon (Zerucha and Ekker 2000). These Dlx^{+} progenitor cells will migrate from the VZ to the SVZ and up into the cortex where they will differentiate into GABAergic interneurons (Eisenstat et al. 1999). The first *Dlx* gene to be expressed in the forebrain is *Dlx2* in immature cells of the VZ and SVZ of the medial and lateral ganglionic eminences (MGE) and (LGE). *Dlx1* expression precedes *Dlx5*/6 in the vast majority of $Dlx2^{+}$ cells of the SVZ and in the mantle zones (MZ) of the MGE and LGE (Eisenstat et al. 1999). *Dlx5* expression precedes that of *Dlx6* and both are expressed in many of the post-mitotic differentiating neurons of the SVZ and MZ (Fig. 1.5) (Wang et al. 2011).

Figure 1.4: Proposed model for the evolution of the *Distal-less* related genes. In recent years, changes in nomenclature encourage the use of *Dlx4* in place of *Dlx7* (Zerucha and Ekker 2000).



Hypothetical common ancestor to nematode, arthropod and vertebrate *Distal-less* genes

As development progresses, *Dlx5*/6 are expressed in the SVZ of the olfactory bulb and at birth in the olfactory tuberculum and neocortex (Merlo et al. 2000). As described above, the four *Dlx* genes are expressed in overlapping domains in the telencephalon and are subsequently activated one after the other suggesting both partial redundant functions and participation in a regulatory cascade (Zerucha and Ekker 2000).

1.6.2 *Dlx* Expression in Other Domains

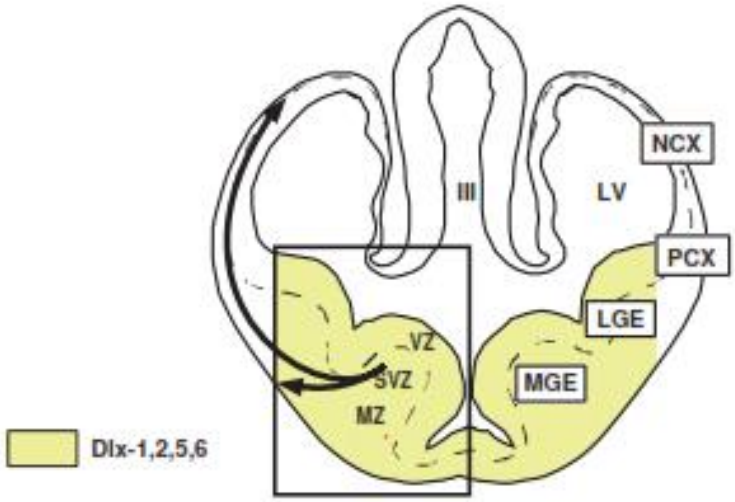
While the *Dlx* genes are expressed in the nervous system of vertebrates, these genes are also expressed in the surface ectoderm including the branchial arches, apical ectodermal ridge of limb buds and in mesodermally derived cells such as otic vesicles, teeth and skeletal tissues (Merlo et al. 2000). In the branchial arches, which harbor many cells from the hindbrain neural crest, *Dlx1/Dlx2* are expressed in all along the proximodistal axis, while the other four *Dlx* genes are expressed more distally. The expression patterns of these genes overlap in the domains they occupy and aid in patterning the proximodistal axis. It has been proposed that because the *Dlx* genes are patterned in such a way, they may play an essential role in jaw patterning of higher vertebrates (Sumiyama and Ruddle 2002). Both *Dlx5* and *Dlx6* are expressed in the perichondrium and osteoblasts of endochondral bones. During vertebrate limb development, all six *Dlx* genes regulate limb outgrowth and are necessary to form the apical ectodermal ridge of the limb bud (Panganiban and Rubenstein 2002).

1.7 Phenotypes of Mutants

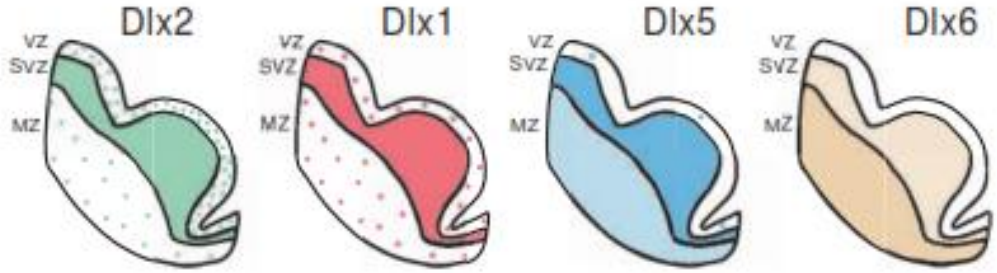
Previous studies of *Dlx* knock-out mice have demonstrated the importance of these genes in patterning early embryos along the proximodistal axis. *Dlx1*/*Dlx2* are expressed

Figure 1.5: A) Transverse section of an E12.5 mouse telencephalon. Arrowheads represent neuronal migration from the subpallium to the cortex (pallium). **B) *Dlx* expression domains in the forebrain**. The black boxed section of the forebrain demonstrates expression domains of *Dlx2* (green), *Dlx1* (red), *Dlx5* (blue) and *Dlx6* (beige). **C) Hypothesized *Dlx* genetic pathway**. *Dlx2* is the first to be expressed, followed by the activation of *Dlx1*, *Dlx5* and *Dlx6* (Adapted from Panganiban and Rubenstein 2002).

A)



B)



C)

Model:

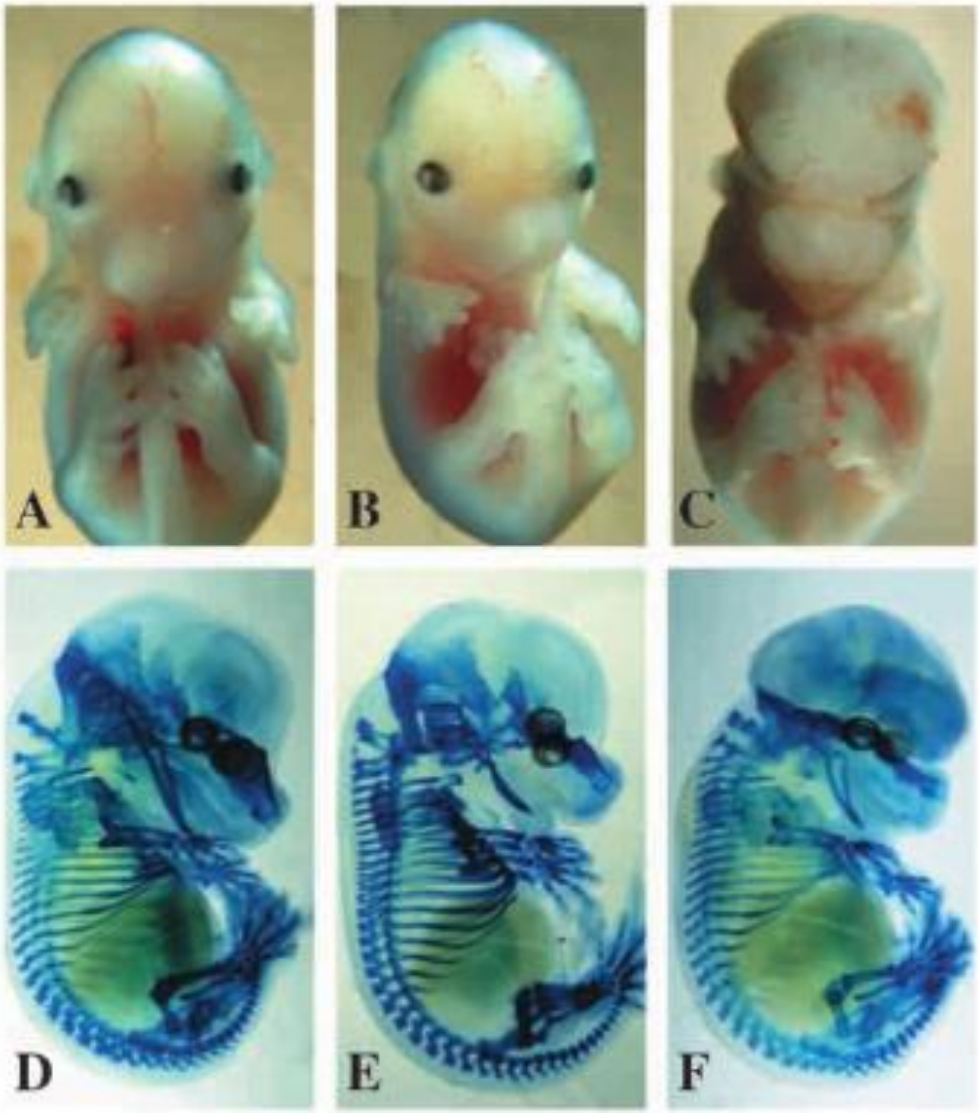
Dlx2 → Dlx1 → Dlx5 → Dlx6

along the proximal axis while the remaining *Dlx* genes are expressed more distally. Mutant *Dlx*2 and *Dlx1/Dlx2* mice are not viable or fertile and die within hours of birth, while single *Dlx1* mutants live up to one month. Craniofacial and enteric nervous system defects seem to be related to the cause of death of these mutants. Phenotypically these mice seem normal, but the internal structures show alterations in the craniofacial bones and in the first and second arches. Double *Dlx1/Dlx2* mutants lack all maxillary upper molars, while the single *Dlx1* and *Dlx2* mutants have normal teeth with all incisors and molars in their correct position (Anderson et al. 1997a). In the forebrain of these mice, disruptions in cell differentiation and migration are prominent in the neocortex resulting in a significant reduction of neurons that use GABA as their chief neurotransmitter (Anderson et al. 1997b). A severe reduction in both *Dlx5* and *Dlx6* transcripts is evident, abolishing almost all of the four *Dlx* genes expressed in the forebrain (Anderson et al. 1997a). Single *Dlx1* mutant mice show no abnormalities in their forebrain, while *Dlx2* mutant mice have a reduction of interneurons in the olfactory bulb (Qiu et al. 1995).

Mice with a targeted mutation in *Dlx3* show a severe phenotype and die before E10.5 due to placental failure. Currently, the function of the remaining *Dlx* genes in the mouse placenta has not been studied and these genes do not compensate for the loss of *Dlx3* function. Mutant mice with a target mutation of the *Dlx4* gene have yet to be identified.

Mutant *Dlx5* mice show a severe phenotype with a variety of craniofacial defects and malformations in the olfactory pit, associated skeletal elements and the inner ear. Approximately 12-28% of these mice lack fusion of the neural tube leading to a cephalic disorder called exencephaly, identified by the development of the brain outside of the skull. Double mutant *Dlx5/Dlx6* mice die at P0 and are very often born with exencephaly. There is also a transformation of the lower jaw into an upper jaw (Depew et al. 2005).

Figure 1.6: Craniofacial defects found in *Dlx5* mutant embryos at E14.5 A) Frontal view of a wild-type embryo. **B)** Frontal view of a *Dlx5* null mutant embryo with a short frontal snout compared to the wild-type embryo in A. **C)** Frontal view of the exencephalic phenotype. **D-F)** Lateral view of E14.5 embryos cartilage skeleton stained with alcian blue. **D)** Side view of a wild-type embryo. **E)** Side view of a homozygous mutant and **F)** Embryo with the exencephalic phenotype (Acampora et al. 1999).

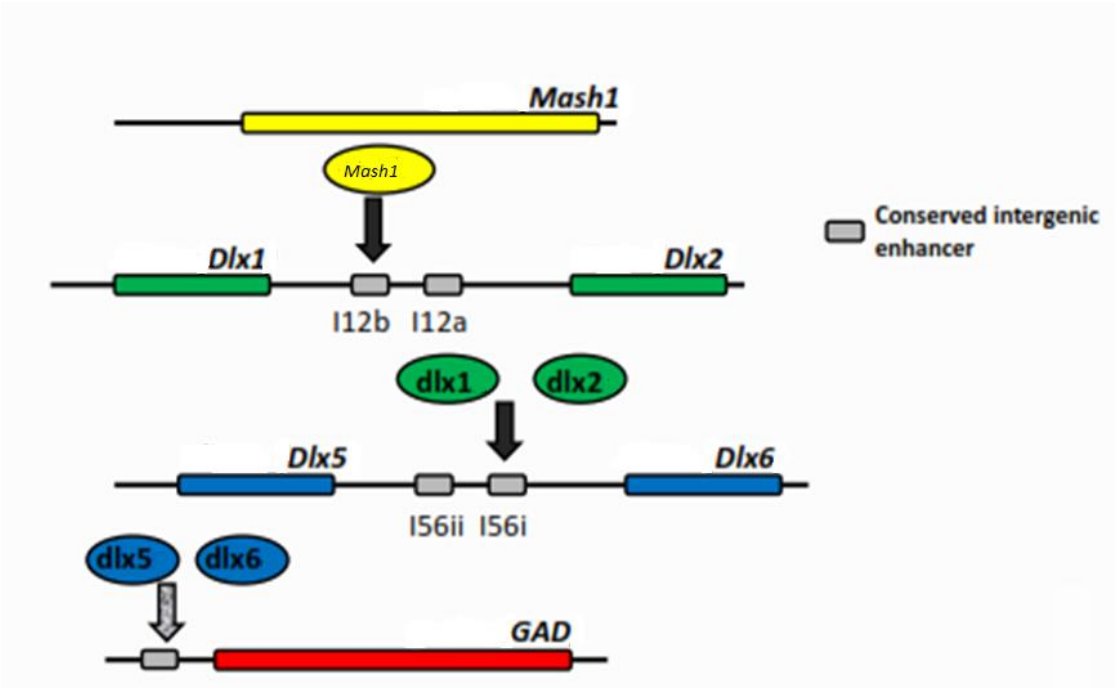

A
B
C
D
E
F

In examining the phenotypic changes that occur in these mutant mice, it is hypothesized that there is functional redundancy between the *Dlx* genes. Their overlapping patterns of expression along with a severe phenotype seen in double mutant mice compared to single *Dlx* mutants suggests this redundancy. It has also been suggested that the *Dlx* genes that are expressed more distally such as *Dlx5/Dlx6* may have compensatory functions for the loss of proximally expressed genes such as *Dlx1/Dlx2*. Functional compensation is seen in the *Dlx1/Dlx2* double mutants in which mutations only have an impact on proximal structures. However, in the case of *Dlx5/Dlx6* double mutants, the more proximal *Dlx1/Dlx2* and *Dlx3/Dlx4* do not compensate for any loss of function, resulting in an extreme phenotype and disruption in the patterning of the proximodistal axis (Panganiban and Rubenstein 2002).

1.8 *Dlx* Regulation

Various genes have been identified as key players in regulating the *Dlx* genes. The *cis*-regulatory elements found within the intergenic regions of each bigene cluster interact with one another and with *Dlx* through enhancer sharing, as a result of the overlapping expression of *Dlx* clusters. The *Dlx* genes act in regulating themselves through interactions with other molecules and enhancers. *Mammalian achaete scute homolog-1* (*Mash1)*, an upstream regulator of *Dlx1/Dlx2* interacts with an enhancer called I12b which is in between the intergenic region of *Dlx1/Dlx2*, initiating transcription of *Dlx1/Dlx2* (Fig.1.7). Both DLX1 and DLX2 have the ability to interact with I56i, activating transcription of *Dlx5* and *Dlx6*. Both DLX5/DLX6 play an activating role on the glutamic acid decarboxylase (Gad) enhancer, which is involved in the production of Gad, an enzyme involved in the synthesis of the inhibitory neurotransmitter GABA (Anderson et al. 1997b). Additional molecules involved in *Dlx* regulation include fibroblast growth factors (FGF's), bone morphogenetic

Figure 1.7: Proposed Gene Regulatory Network of the *Dlx* genes: *Mash1* regulates *Dlx1/2* through the I12b enhancer, initiating transcription of *Dlx1/Dlx2*, whose transcripts bind to I56i, initiating transcription of *Dlx5/Dlx6*. Dlx5/Dlx6 transcripts bind to the Gad enhancer, initiating transcription of *Gad* (Courtesy of Jacob Pollack).


Mash1
Mash1
Conserved intergenic enhancer
Dlx1
Dlx2
I12b
I12a
dlx1
dlx2
Dlx5
Dlx6
I56ii
I56i
dlx5
dlx6
GAD

protein 4 (BMP4), retinoic acid and the sonic hedgehog (Shh) signaling pathway, which induces expression of *Dlx* in the forebrain (Panganiban and Rubenstein 2002). Lastly, in the regulation of the *Dlx* genes, there are both *cis* and *trans* acting factors that bind to these genes, playing a role in activating transcription. Additionally, non-coding RNA's (ncRNA's) such as *Embryonic ventral forebrain-2* (*Evf*-2) are also involved in the regulation of the *Dlx* genes, playing a unique role. The *Evf-2* gene has been placed into a new class of ncRNA's called transcription-regulating ultraconserved ncRNAs (trucRNAs) and is involved in gene regulation as well as the development of GABAergic interneurons (Feng et al. 2006)

1.9 *Cis*-Regulatory Elements at *Dlx* Loci

The three vertebrate *Dlx* bigene clusters possess highly conserved sequences or *cis*-acting regulatory elements within their short (2.5-10 kb) intergenic regions that may act as potential sites of activity for a variety of regulatory factors (Ghanem et al. 2003). Two short 400bp and 300bp sequences named I56i and I56ii have been identified within the intergenic region of *Dlx5* and *Dlx6*. The mouse I56i sequence is 100% identical to its human counterpart except for a 3bp insertion in the human sequence, while I56ii has 98% sequence identity between human and mouse (Ghanem et al. 2003). The I56i enhancer, the main objective of this thesis, will be described in greater details in section 1.11. The I56ii enhancer is active starting at E.10.5 in the telencephalon, diencephalon and apical ectodermal ridge (Ghanem et al. 2003).

Two highly conserved sequences I12a (550bp) and I12b (400bp) have been identified within the intergenic region of *Dlx1/Dlx2*. In the mouse, the activity of the I12b element begins at E.10 in the telencephalon, diencephalon and apical ectodermal ridge. In contrast to the I12b enhancer, the I12a element is not active in the forebrain but rather in the

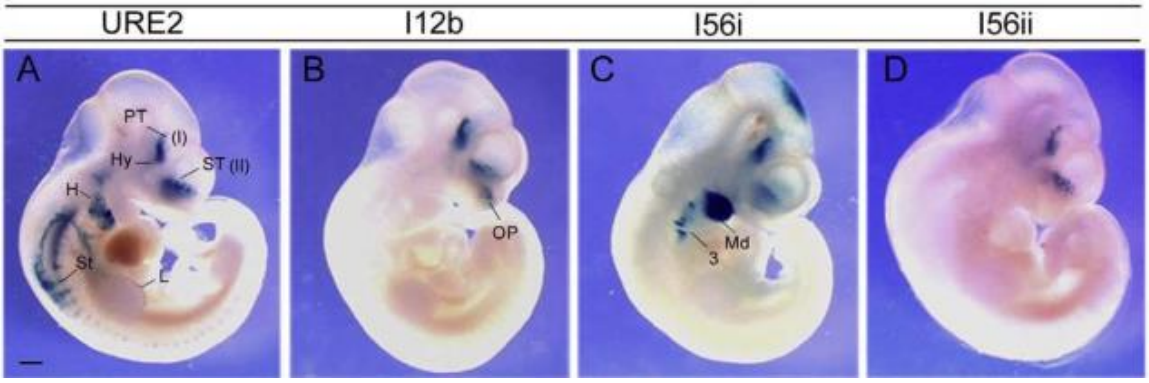
mesenchyme of the first branchial arch and hyoid arch beginning at E9.5 until E16.5 (Ghanem et al. 2003). Upstream from the *Dlx1/Dlx2* locus are URE1 and URE2. Upstream regulatory element 2 (URE2) is active starting at E.11 in the subpallial telencephalon, prethalamus and hypothalamus as well as in the somites and apical ectodermal ridge (Ghanem et al. 2007). Although the forebrain enhancers have overlapping activities, they have very little sequence similarity, indicating the possibility that they may be responding to distinct trans-acting factors (Poitras et al. 2007). The 437bp I12b enhancer is located between the *Dlx1/Dlx2* genes and has many characteristics similar to the I56i enhancer. It is active in the majority of the same cells as I56i including the LGE, MGE, caudal ganglionic eminence (CGE) and MZ. The overlapping activities of I56i and I12b raise the possibility of the two enhancers responding to similar regulatory factors (Fig 1.8) (Ghanem et al. 2007). Through DNase I footprinting, six binding sites that are potentially bound by transcription factors were identified in the I12b sequence. Co-transfection assay experiments suggest that the DLX proteins can activate transcription through these binding sites and affinity for binding is dependent on the presence of the homeodomain. An important homeodomain transcription factor, *Mash1 (ASCL1)* is responsible for the production of neuronal precursor cells and directly binds to I12b, playing an essential role in forebrain regulation (Ghanem et al. 2007). Characterization of knock-out mice harboring the I12b enhancer deletion revealed a decrease in both *Dlx1/2* transcript levels which could inhibit cell proliferation in the developing mouse forebrain. *Dlx5/6* transcript levels remained stable compared to the wild-type mouse while *Mash1*, the upstream regulator of *Dlx1/2* has increased mRNA levels.

1.10 *Evf-2* as a Non-Coding RNA

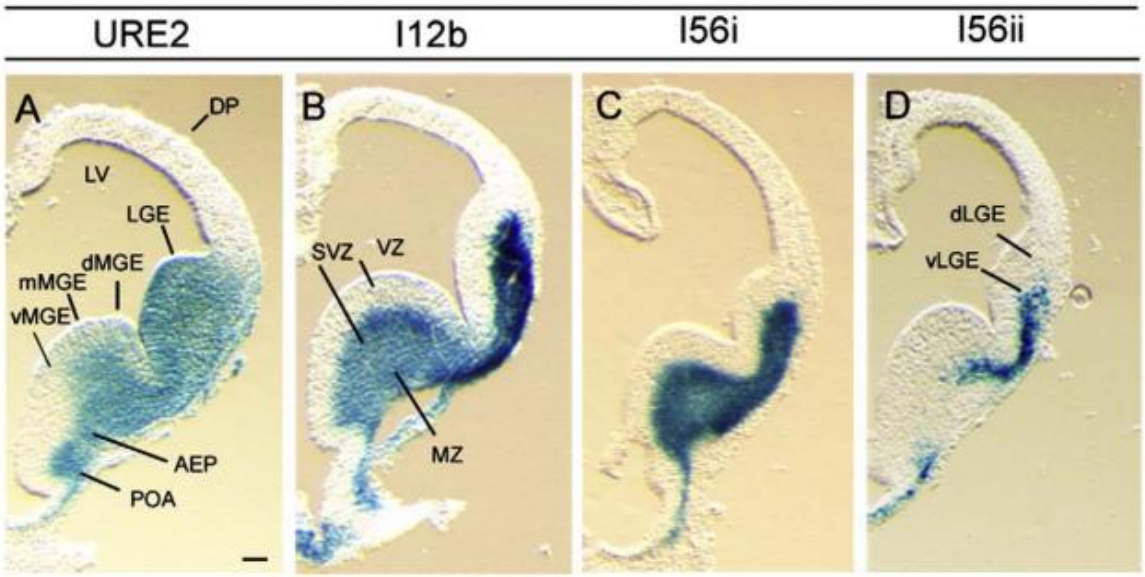
The discovery of an ultraconserved noncoding sequence *Evf-2* has enforced the

Figure 1.8: Domains of activity of the vertebrate *Dlx* forebrain enhancers. Panel 1) A-D) Activities of URE2, I12b, I56i and I56ii in E10.5 transgenic mouse embryos showing expression of the *lacZ* reporter gene. **Panel 2) A-D)** Coronal hemisections showing lac*Z* expression under the control of URE2, I12b, I56i and I56ii in the subpallial telencephalon in E10.5 transgenic mouse embryos (Ghanem et al. 2008).

Panel 1)



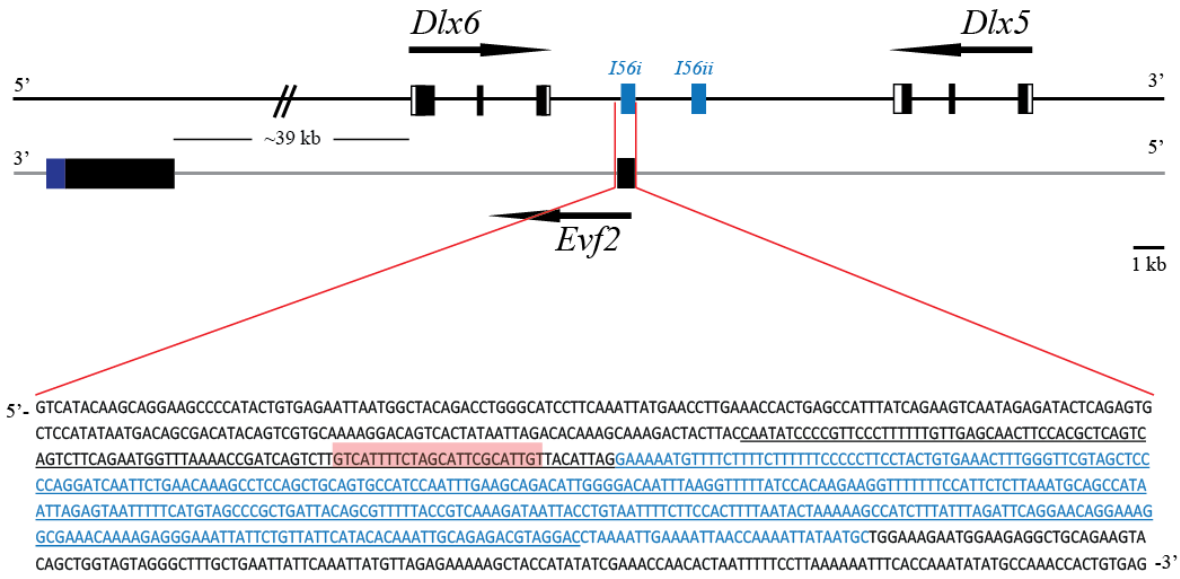
Panel 2)



importance of studying non-coding RNA's involvement in the regulation of developmental genes. Within the non-coding intergenic region of *Dlx5/Dlx6*, the I56i enhancer has been shown to transcribe a small part of the second exon of *Evf-2* (Fig. 1.9). This non-coding RNA is an alternatively spliced form of *Evf-1*, a novel downstream target of the patterning protein, sonic hedgehog (Feng et al 2006). The expression pattern of *Evf-2* overlaps endogenous I56i activity in the ventral forebrain and is expressed in the telencephalic region of the LGE, MGE and in the immature neurons migrating from the VZ (Feng et al. 2006). Using reporter assays, it was shown that single-stranded sense *Evf-2* RNA increases reporter activity of both conserved enhancers (I56i and I56ii) within the *Dlx5/6* intergenic region when cooperating with DLX proteins. Immunoprecipitation experiments have demonstrated that *Evf*-2 and DLX2 form a stable complex in vivo, stabilizing the interaction between DLX2 and *Dlx5*/*Dlx6* enhancers to ultimately increase transcriptional activity through trans-acting cooperativity (Feng et al. 2006). *Evf*-2 is required for DLX protein recruitment to both I56i and I56ii. This was confirmed through chromatin immunoprecipitation (ChIP) and qPCR on E13.5 MGE chromatin of wild-type and *Evf-2* mutants, confirming that in *Evf-2* mutant chromatin, DLX proteins do not bind to either *Dlx5/Dlx6* enhancer (Bond et al. 2009).

In determining the role of *Evf-2 in vivo*, loss-of-function mice have been generated and shown to be viable, fertile and had no obvious morphological abnormalities compared to wild-type controls. Mice that lack a functioning *Evf*-2 gene have increased transcript levels of both *Dlx5*/*Dlx6* and *Dlx2* remains stable, suggesting a negative transcription regulating role *in vivo* (Bond et al 2009). The absence of *Evf-2* also has an effect on GABAergic interneuron precursors migrating towards the hippocampus and denate gyrus (Bond et al 2009.) *In situ* hybridization confirmed a decrease of *Gad1*, while immunohistochemistry

Figure 1.9: Schematic representation of mouse *Evf-2* genomic organization. This schematic demonstrates the overlapping region between *Evf-2* and the I56i enhancer (blue) located in the intergenic region of *Dlx5/6* cluster on the positive strand. Sequence in blue represents the genomic sequence of the I56i enhancer and the underlined sequence corresponds to the first exon of *Evf-2*. Exons and UTRs are represented in black and white respectively and the purple region represents the *Evf-2* unique region located on the 3' end of the second exon. Scale bar = 1 kb (Courtesy of Siavash Fazel-Darbandi).



5'- GTCATACAAGCAGGAAGCCCCATACTGTGAGAATTAATGGCTACAGACCTGGGCATCCTTCAAATTATGAACCTTGAAACCACTGAGCCATTTATCAGAAGTCAATAGAGATACTCAGAGTG
CTCCATATAATGACAGCGACATACAGTCGTGCAAAAGGACAGTCACTATAATTAGACACAAAGCAAAGACTACTTACCAATATCCCCGTTCCCTTTTTTGTTGAGCAACTTCCACGCTCAGTC
AGTCTTCAGAATGGTTTAAAACCGATCAGTCTTGTCATTTTCTAGCATTCGCATTGTTACATTAGGAAAAATGTTTTCTTTTCTTTTTTCCCCCTTCCTACTGTGAAACTTTGGGTTCGTAGCTCC
CCAGGATCAATTCTGAACAAAGCCTCCAGCTGCAGTGCCATCCAATTTGAAGCAGACATTGGGGACAATTTAAGGTTTTTATCCACAAGAAGGTTTTTTTCCATTCTCTTAAATGCAGCCATA
ATTAGAGTAATTTTTCATGTAGCCCGCTGATTACAGCGTTTTTACCGTCAAAGATAATTACCTGTAATTTTCTTCCACTTTTAATACTAAAAAGCCATCTTTATTTAGATTCAGGAACAGGAAAG
GCGAAACAAAAGAGGGAAATTATTCTGTTATTCATACACAAATTGCAGAGACGTAGGACCTAAAATTGAAAATTAACCAAAATTATAATGCTGGAAAGAATGGAAGAGGCTGCAGAAGTA
CAGCTGGTAGTAGGGCTTTGCTGAATTATTCAAATTATGTTAGAGAAAAAGCTACCATATATCGAAACCAACACTAATTTTTCCTTAAAAAATTTCACCAAATATATGCCAAACCACTGTGAG -3'

demonstrated an accompanying decrease of GABA, the primary inhibitory neurotransmitter for GABAergic neurons. In these mice, the number of GABAergic interneurons decreases by approximately 65% in the hippocampus and denate gyrus. Rescue experiments involving electroporation of *Evf-2* in the mutant mice restored *Gad1* levels by approximately 30%, suggesting that *Evf*-2 may play a role in *Gad1* activation through trans-acting mechanisms (Bond et al. 2009). However, in *Evf*-2 mutant adult forebrain, *Gad1* transcript levels and number of GABAergic interneurons were comparable to wild-type mice, suggesting that the mutant mice have the ability to recover to normal levels around two months into development, approximately the same time *Evf*-2 begins to become down-regulated (Bond et al. 2009).

1.11 The Vertebrate *Dlx* I56i Forebrain Enhancer

Previous experiments from Zerucha et al. have identified a conserved sequence between the zebrafish *dlx5a/dlx6a* intergenic region and its mouse ortholog *Dlx5/Dlx6.* They found a 1.4kb *Xho*I±*Eco*RI fragment from the zebrafish *dlx5a/dlx6a* intergenic region that was able to hybridize to restriction fragments with the mouse *Dlx5/Dlx6* intergenic region. This sequence, called I56i was analyzed and found to be 450bp in length and highly conserved between both zebrafish and mouse (Zerucha et al. 2000). Generating a construct containing a *lacZ* reporter gene has shown that the activity of I56i starts at approximately E.10 and lasts past birth. The activity of this enhancer extends to the forebrain cells of the ventral thalamus, hypothalamus, subpallial telencephalon and diencephalon which strongly overlap expression of endogenous *Dlx* in the mouse. Other domains of activity include the branchial arches and the apical ectodermal ridge (Zerucha et al. 2000). It has been hypothesized that the I56i element may act as a player in cross regulation of neighboring *Dlx*

genes. The I56i element has two putative DLX binding sites and it is suggested that this enhancer plays a regulatory role involved in modulating expression of *Dlx5* and *Dlx6* by *Dlx1* and *Dlx2*. Through ChIP experiments, it has been demonstrated that both DLX1 and DLX2 have the ability to bind to one or both of this enhancers binding sites (Zhou et al. 2004). When either of the DLX binding sites on the I56i enhancer is altered, enhancer activity decreases as a result of reduced ability of DLX2 and/or DLX1 to bind and activate transcription of *Dlx5* and *Dlx6*. In *Dlx1/Dlx2* double mutants I56i activity decreases, resulting in abnormal neuronal migration which further supports the importance of DLX1/DLX2 in activating I56i and ultimately initiating transcription of the *Dlx5/Dlx6* locus (Zerucha et al. 2000).

1.11.1 An SNP in the *Dlx* I56i Forebrain Enhancer

The *Dlx* genes have been shown to play essential roles in the development of precursor cells that give rise to the various subtypes of GABA interneurons of the forebrain. Disruptions in the development of these interneurons have been linked to neurological disorders including epilepsy, schizophrenia and autism (Poitras et al. 2010). In patients with autistic spectrum disorder (ASD), linkage studies have identified susceptibility loci on chromosome 2q and 7q, the same chromosomes that harbor *Dlx1/Dlx2* and *Dlx5/Dlx6*. The *Dlx* exons, exon-intron boundaries along with their regulatory elements (URE2, I12a, I12b, I56i and I56ii) were sequenced in 161 autism probands taken from the Autism Genetic Resource Exchange Collection and compared to 58 non-autistic siblings as well as 188 non-autistic individuals (Hamilton et al. 2005). Sequencing results revealed 33 variants, comprised of 31 single nucleotide polymorphisms (SNP) and 2 deletion/insertion polymorphisms. Four SNPs were identified in URE2, 1 in I12b and 1 in the I56i enhancer

(Hamilton et al. 2005). The SNP in the I56i enhancer is of interest because of its location in an ultraconserved sequence which is not usually predisposed to variation. Poitra et al. 2010 have provided evidence that a single nucleotide change in the center of the I56i enhancer is sufficient in reducing the activity of I56i in the medial and caudal eminences of the forebrain as well as in the GABAergic interneurons of the somatosensory cortex. The affinity of the DLX proteins for their binding sites *in vitro* was significantly reduced along with the activation of the I56i enhancer by DLX proteins.

In determining the functional consequence of an SNP in the I56i enhancer, "knock-in" mice were generated and subjected to a variety of behavioral tests including juvenile interaction and adult social interaction testing. The results showed no changes in behavioral phenotypes between the wild-type and vI56i mice. It is suggested that the functional consequences of the SNP in the I56i enhancer may have implications in the developmental abnormalities associated with neurological disorders (Poitras et al. 2010).

2 Statement of Inquiry

The *distal-less homeobox* (*Dlx*) genes are homologs of the primitive developmental gene *distal-less (Dll),* the earliest known gene expressed in developing insect limbs. The first known function of the *Dlx* genes was appendage development. In more recent years, research has demonstrated the significance of these genes in a variety of other developmental processes including skeletal and sensory organ development as well as neurogenesis of the forebrain. The vertebrate *Dlx* genes encode homeodomain transcription factors belonging to convergently transcribing bigene pairs, each of which are expressed in overlapping cell populations in the developing forebrain.

In the forebrain, four of the six *Dlx* genes are expressed (*Dlx1/2* and *Dlx5/6)* and are

critical players in neurogenesis, particularly in GABAergic neuron proliferation, migration and survival. The spatial and temporal expression pattern of the *Dlx* genes in the forebrain overlaps cell populations that are positive for *Gad*, the gene responsible for transcribing enzymes that synthesize GABA, the chief inhibitory neurotransmitter of the brain.

Abnormal development of the various subtypes of GABAergic neurons has been associated with neurological disorders including autism spectrum disorder, rett syndrome and anxiety. Since the expression patterns of the *Dlx* genes overlap those of both *Gad* and GABA any dysfunction in these genes may have implications in neurological disorders.

The precise regulatory network controlling the *Dlx* genes is not fully characterized but it has been established that these transcription factors interact with each other and with multiple conserved regulatory elements to regulate transcription. The Ekker laboratory has been successful in identifying four regulatory elements known to take part in regulating the *Dlx* genes. These elements are URE2 and I12b located in the *Dlx1/Dlx2* loci and I56i and I56ii in the *Dlx5/Dlx6* loci. While the complete regulatory mechanisms of these enhancers are not fully understood, our laboratory has shown, using transgenic mice that the activity of these enhancers overlaps with endogenous *Dlx* expression in the forebrain.

The objective of my research was to characterize the functional consequences in mice that harbor one or multiple *Dlx* enhancer deletions. In characterizing mutant mice with one or more enhancer deletion(s) I have addressed numerous questions including 1) Does the absence of one or more enhancer contribute to changes in the morphology or size of the developing mouse? 2) Does the absence of *Dlx* enhancer(s) contribute to a change in *Dlx* function? 3) What are the possible impacts of the loss of an enhancer(s) on the expression pattern of the *Dlx* genes in the developing forebrain? 4) Is the differentiation of various subtypes of GABA-expressing neurons compromised due to the absence of a *Dlx* enhancer?

5) Is there a behavioral phenotype in mutant mice that harbor the I56i enhancer deletion?

In addressing the above questions I have successfully characterized the functional changes that occur in various mice lines that harbor either one or multiple *Dlx* enhancer deletions. This research highlights the importance of *cis*-regulatory elements in regulating *Dlx* function and demonstrates that the lack of the I56i enhancer contributes to a behavioral phenotype in which the mutant mice are more anxious and have learning deficiencies related to defects in the amygdala.

3 Materials and Methods

3.1 Animal Maintenance

Mice were housed in a sterile environment in the Roger Guidon Hall and Vanier Mouse Facility at the University of Ottawa with a 12 hour light/dark cycle. Mouse colonies were given unlimited access to water and fed a standard mouse chow. Personnel working with mice received certification through the National Institute for Animal User Care training program. Experimental protocols were approved by the University of Ottawa's Animal Care Ethics Committee.

3.2 Generation of I56i Knockout Mice

Mutant ΔI56i mice were generated at the Transgenic Mouse Core Facility at the McGill Cancer Center. The strategy and target vector used to produce mutant ΔI56i mice is depicted in Fig. 3.1. Through homologous recombination, a LoxP-flanked PGK-neomycin-resistant cassette replaced the entire I56i enhancer on a Bacterial Artificial Chromosome (BAC) harboring the *Dlx5/Dlx6* locus. The BAC was screened and sequenced to ensure recombination occurred. The vector was electroporated into 129Sv mouse embryonic stem (ES) cells and positive clones were selected with gentamicin. ES cells were screened for the

presence of the neomycin cassette through quantitative real-time PCR and a positive ES clone was injected into a host C57BL/6 blastocyst to generate chimeric mice. Chimeras were selectively mated with C57BL/6 wild-type mice and genotyped for the production of heterozygote progeny that were positive for the I56i deletion in their germ line. Once mice heterozygous for the I56i deletion were established, we mated two heterozygotes to produce mutant ΔI56i mice.

3.3 Genomic DNA Extraction

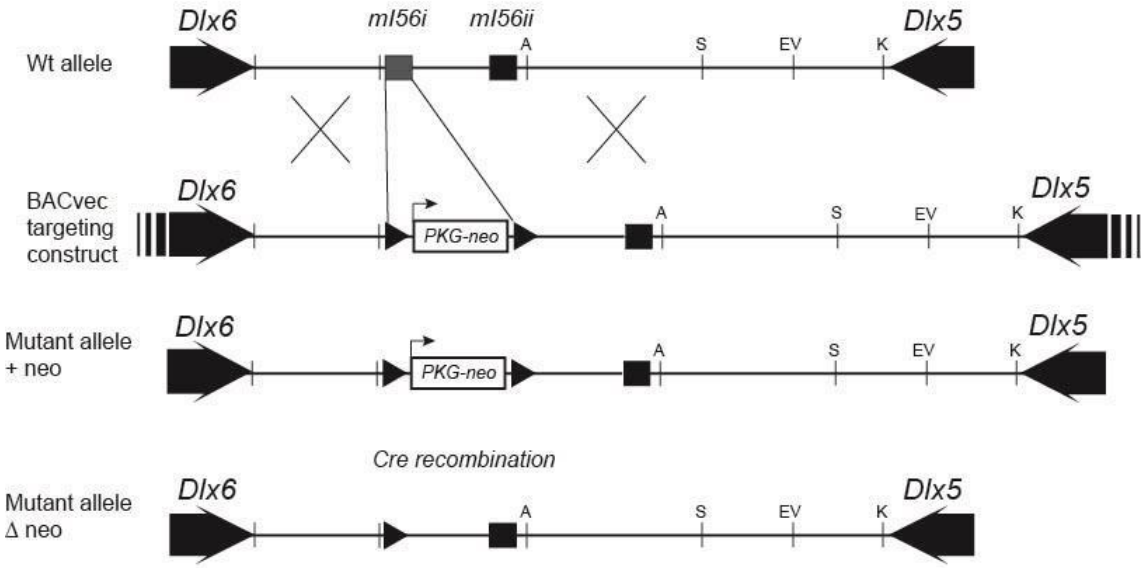
Tail clippings were taken from 3 week old mice following weaning from their mother. Tissue samples were digested for a minimum of 2 hours at 55^0C in 270µL digestion buffer (100mM NaCl, 1% SDS, 100mM EDTA and 50mM Tris-HCl pH 8.0) and 30 µL of Protinase K solution [10mg/mL]. Following digestion, 0.05 times the volume of NaCl was added to the disintegrated tissue and centrifuged at 15,800 g for 5 minutes. The supernatant was isolated and 0.8 times the volume of isopropanol was added to precipitate genomic DNA. The DNA pellet was isolated and washed with 300 µL of 70% ethanol. The washed pellet was air dried and re-suspended in 300 µL of TE buffer (100mM Tris-HCl pH 8.0 and 10mM EDTA). Extracted genomic DNA was stored at 4^0C up to two years.

3.4 Genotyping

Mice harboring the I56i enhancer deletion on one or both alleles were screened using the polymerase chain reaction and primers designed to amplify the presence of a neomycin cassette and the I56i enhancer. Individual PCR reactions consisted of 2µL of genomic DNA (~200ng), 10x PCR buffer (100mm TrisHCl pH8.3, 500mM KCl, 12mM MgCl2)**,** dNTPS [0.2mM], forward and reverse primers [10pmol] and 1 unit of Taq Polymerase. The following PCR protocol was used on the thermal cycler for all genotyping experiments: One

Figure 3.1: Targeting strategy for the deletion of the I56i enhancer in mouse embryonic stem cells. The I56i BAC targeting vector containing a neomycin cassette flanked by loxP sites (black triangles) undergoes homologous recombination with one allele of the endogenous I56i CRE (grey box). Homologous recombination regions, represented by cross lines result in the replacement of the ~450bp enhancer with the neo cassette. The black arrows represent the transcriptional orientation of *Dlx5* and *Dlx6* genes.

mI56i deletion



cycle at 95^0C for 4 minutes, 30 cycles of (95°C for 1min, 58°C for 1min, 72°C for 2min), and a 72°C 5 minute extension followed by 4°C to cool the reactions. The PCR products were run on a 1% agarose gel and analyzed under an ultraviolet light. The presence of a neomycin cassette band and no I56i band was indicative of a mouse homozygous for the enhancer deletion. Genomic DNA that amplified both Neo and a primer specific region of the I56i enhancer was indicative of a mouse heterozygous for the I56i deletion. Wild-type mice did not have a neomycin band and only a band for the I56i enhancer was seen. Mice that harbored two enhancer deletions were screened in the same manner as the I56i knockout mice. Mutant mice with vI56i the absence of the 12b enhancer were screened using the vI56i and I12b primer sequences identified below. The presence of a 383bp vI56i band is indicative of the presence of a single nucleotide polymorphism of an adenine for guanine located midway of the I56i enhancer and a 300bp I12b band was indicative that the mouse was homozygous for both the vI56i and absence of the I12b. Mutant mice harboring the I56i and I12b deletion were screened using primers for I56iΔNeo and ΔI12b. The presence of a 404bp I56i band and a 300bp I12b band was indicative of a mouse that was homozygous for both the I56i and I12b deletion. For all mouse lines genotyped, Fetal Hemoglobin (FH) was used as a positive control. The primer sequences used for genotyping are as follows:

Neo.Fwd 5’ TCTCCTGCCGAGAAAGTAT 3’

Neo.Rev 5’ CAACAGATGGCTGGCAACTA 3’

FH.Fwd 5’ GATCATGACCGCCGTAGG 3’

FH.Rev 5’ CATGAACTTGTCCCAGGCTT 3’

I56i.Fwd 5’ CCAGCTGCAGTGCCATCC 3’

I56i.Rev 5’ TTAGGTCCTACGTCTCTGCA 3’

I56iΔNeo.Fwd 5’ TGCTCCTGCCGAGAAAGTAT 3’

I56iΔNeo.Rev 5’ CAACAGATGGCTGGCAACTA 3’

vI56i.Fwd 5’ CCCCAATGTCTGCTTCAAAT 3’

vI56i.Rev 5’ GGAAGCCCCATACTGTGAGA 3’

I12b.Fwd 5’ TGAGTCTGTAATGGCAAAATGC 3’

I12b.REV 5’ CAGGTGCAGATTCCCTGAAG 3’

3.5 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from dissected tissue of the ventral telencephalon of mice. Total RNA was extracted using the QIAGEN purification of total RNA kit. RnaseZap (Ambion) was used throughout all RNA extraction procedures and the final RNA concentration was measured using a Nanodrop spectrophotometer (Thermo Scientific). Total RNA (2µg) was synthesized into single-stranded cDNA using random hexamers and SuperScript II Reverse Transcriptase (Invitrogen). Quantitative Real-Time PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). For each gene tested, a total of six biological replicates were tested in triplicates. Standard curves for each gene were produced to quantify mRNA copy number. Each individual qPCR reaction contained 1µL of a 1:10 dilution of cDNA, 7.5µL of SYBR Green PCR master mix (Qiagen), 0.5µL of 10pmol of each forward and reverse primer and 4.5µL of ddH_2O. *Elongation factor 1-alpha (Ef1-a)* was used as an internal control and all reactions were amplified with a 10 minute initial hot start activation at 95^oC followed by 40 cycles of (30 s at 95°C, 1 min at 55°C, and 1 min at 72°C). At the end of each assay, a melt curve was produced to ensure only one specific product was amplified. All statistical calculations were based off of the Pfaffl method of analyzing qPCR data (Pfaffl 2001). All data was calculated using Microsoft Excel 2010. Significant changes in transcript levels were calculated using a

Student's t-test with a P value <0.05 being considered as statistically significant. The sequences for all genes assayed are as follows:

Ef1α.Fwd 5' AAGCTCTTCCTGGGGACAAT 3'

Ef1α.Rev 5' ATGCTATGTGGGCTGTGTGA 3'

Dlx1.Fwd 5' CAGTTGCAGGCTTTG 3'

Dlx1.Rev 5' ACTTGGAGCGTTTGTTCTGG 3'

Dlx2.Fwd 5' GCCTCACCCAAACTCAGG 3'

Dlx2.Rev 5' GCCGCTTTTCCACATCTTC 3'

Dlx5.Fwd 5' CGACTTCCAAGCTCCGTTC 3'

Dlx5.Rev 5' TTCTTTCTCTGGCTGGCTG 3'

Dlx6.Fwd 5' CGGACCATTTATTCCAGCC 3'

Dlx6.Rev 5' CGCTTATTCTGAAACCATATC 3'

Gad1.Fwd 5' AGCAGATCCTGGTTGACTGT 3'

Gad1.Rev 5' TCAGCCATTCACCAGCTAAA 3'

Gad2.Fwd 5' TCATTGCCCGCTATAAGATG 3'

Gad2.Rev 5' GCAGCTCCCTTCTTGAGAGA 3'

Evf-2.Fwd 5' CAATGCGAATGCTAGAAAATGA 3'

Evf-2.Rev 5' ACAGCAGTGGGAAAGCAATC 3'

3.6 Collection of Murine Tissue and Sectioning

Mouse embryos were extracted from female mice that were heterozygous for the I56i deletion that had mated with heterozygous males. Selective mating's were set mid-afternoon and the following morning the female was checked for the presence of a vaginal plug, indicative that the mice had successfully mated. If there was no vaginal plug, the mice were left to mate for an additional four days, checking for a plug every morning. The presence of a plug is the start of embryonic day 0.5 after conception, E0.5. Pregnant females carrying

embryos at developmental stages E.11.5, E14.5 and P0 were anesthetized through CO_2 exposure followed by cervical dislocation. Embryos were dissected and placed in 1x PBS buffer. Tissue from the embryos tail was removed and used for genomic DNA extraction, while the brains were removed and fixed in 4% paraformaldehyde (PFA) in 1x PBS solution overnight at 4^0C. The following day, brains were washed 3 times in 1x PBS solution and soaked in 30% sucrose in 1x PBS overnight at 4^0C. Following calibration in sucrose, the brains were embedded in cryostat molds using OCT compound and snap frozen in liquid nitrogen at -80^0C. Frozen cryostat blocks were set to a temperature of -20^0C in the sectioning Leica CM1850 chamber for 1 hour. Brain sections were then cut to a thickness of 20 µm and collected on Fisher Superfrost/Plus microscope slides and stored at -80^0C until further use.

3.7 *In situ* Hybridization on Mouse Forebrain Sections

Antisense RNA probes were labeled with Digoxygenin-dNTP and synthesized from a variety of cDNA clones generated from previous Ekker lab members. The vector that contained the cDNA clone was linearized with the appropriate enzyme and the antisense riboprobe was synthesized using either the T7, T3 or Sp6 polymerase (Table 3.1). Brain sections stored at -80^oC were thawed at room temperature for two hours before beginning hybridization. Diluted probes were added to 1mL of hybridization buffer [50% formamide, 10% dextran sulfate, yeast tRNA 1mg/mL, 1x Denhardts solution, 1x ddH_20 and 1x salt solution (0.2 M NaCl, 10 mM Tris-HCl, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 1 mM Tris-base, 5 mM EDTA pH7.5)]. Hybridization mixture was heated for 10 minutes at 70^oC to denature the probes. Approximately 500µL of hybridization mixture were added to each slide and each slide was placed in a sealed humidified chamber lined with paper towel soaked in old solution A (1×SSC, 50% formamide, and 0.1% Tween 20) and incubated overnight at 70^oC. Slides

were transferred to Coplin jars containing fresh solution A and placed in a 70^{o}C water bath for 2x 30 min washes. Slides were then transferred to new Coplin jars and washed at room temperature in 1x TBST (1.4M NaCl, 27mM KCl, 0.25M Tris HCl and 1% Tween 20) and incubated for 2x 30 minute washes. Slides were removed from Coplin jars and placed in a humidified chamber lined with paper towel soaked in distilled water. On each slide, 500µL of blocking solution (10% heat-inactivated sheep serum in 1x TBST) was added and incubated at room temperature for three hours. Anti-Dig AP Fab fragment (Roche) diluted 1:2000 in 1mL of blocking solution and 500µL was added to each slide and incubated overnight in the same humidified chamber at 4^{o}C. In the morning, slides were transferred to Coplin jars and washed at RT 5x in 1 x TBST for 20 min. Slides were then washed 2 x 10 minutes with 1 x NTMT (100 mM NaCl, 100 mM Tris HCL pH 9.5, 50 mM $MgCl_2$, and 0.1% Tween 20) at RT. Slides were laid flat to dry and stained at RT with 500µL of NTMT containing 4.5µL NBT (nitrobluetetrazolium chloride) and 3.5µL BCIP (5-bromo-4-chloro-3-indolyl-phosphate) for ~1-2 hours. The staining reaction was stopped by washing slides 2x 5 minutes in dd$H_2$0. Slides were fixed by incubating them with 400µL of 4% PFA in 1xPBS for 20 minutes. Following final fixation, slides were allowed to dry before mounted with coverslips using Aquatex mounting media (EMD Chemicals).

3.8 Immunohistochemistry on P35 Mouse Forebrain Sections

Mouse forebrain sections were air dried at RT for 1 hour and placed in a Tupperware box with the bottom soaked with wet paper towel to produce a moist environment. PBT was diluted to a 0.1% solution and 500µL were added to each slide for 10 minutes. Slides were incubated for 2 hrs with 500uL of blocking solution (1% bovine serum albumin, 10% calf serum and 0.1% Tween 20).Slides were placed in Coplin Jars containing 1x PBS for 10

Table 3.1: cDNA clones used to synthesize mRNA antisense probes for *in situ* hybridization of mutant ΔI56i embryonic forebrain tissue.

cDNA clone	Vector	Linearization Enzyme	RNA Polymerase	Probe Length (Kb)	Probe Concentration
mouse Dlx1	PBS-SK	BamHI	T3	0.24	1.5ng/mL
mouse Dlx2	PBS-SK	HindIII	T3	1.7	1.5ng/mL
mouse Dlx5	PBS-SK pCR2.1	SmaI/SalI	T3	1.6	1.5ng/mL
mouse Dlx6	TOPO	SpeI	T7	0.37	1.5ng/mL
mouse Evf-2	pDrive	BamHI	Sp6	0.944	1.5ng/mL
mouse Gad2	PBS-SK	BamHI	T3	0.88	1.5ng/mL

minutes at RT. Primary antibodies were added to blocking solution and 500µL of this solution were added to the slides which were allowed to incubate overnight at 4^0C. On the second day, slides were washed three times in 1X PBS for ten minutes. The secondary antibodies were diluted 1:1000 in blocking solution and 500µL of this solution were added to the slides which were placed in a dark seal Tupperware for 2 hours. Following two hours, the slides were washed three times in 1X PBS for ten minutes at RT. The slides were allowed to air dry in the dark and were mounted with Aqua Poly/Mount (Polysciences Inc.) All slides were left in the dark at 4^0C until imaged using a Zeiss Axiophot fluorescent microscope. Primary antibodies used and their dilution in blocking solution are as follows; rabbit polyclonal anti-GABA (1:5000), rabbit polyclonal anti-calbindin (1:1000), mouse polyclonal anti-parvalbumin (1:1000).

3.9 Mouse Behavioral Testing

All behavioral testing was completed at the Mouse Behavioral Core Facility at the Roger Guidon Hall, University of Ottawa Campus. All protocols used were based off of standard tests approved by the Behavioral Core Facility, Ottawa ON.

3.9.1 Beam Break

The beam break is a test that measures general motor activity of mice. Mice are placed in a housing cage that is stationed on a metal frame connected to infrared receptors and emitters with a Micromax analyzer (Accuscan). The mice are free to roam the cage for two hours and their motor activity is tracked by invisible infrared light beams. Photocell emitters located on each side of the cages send horizontal beams of infrared light to the opposite side of the cage and are detected by photocell receptors. As the animal moves

through the cage and breaks the beams, the photocell analyzer records the average number of beam breaks over a two hour period.

3.9.2 Elevated Plus Maze

The elevated plus maze is a standard test that measures fear and anxiety based on the preference of the animal to explore dark enclosed spaces compared to bright exposed places. The mice are placed one at a time in the center of an elevated four arm maze measuring 5cm wide and 60cm long. Two of the arms are open platforms while the other two have enclosed walls measuring 14.5cm high. The maze is elevated one meter above ground and the test is performed with the lights on to increase the anxiety of being in an open arm. Once the mice have been placed in the center of the maze they are free to explore for 10 minutes while the investigator leaves the room. The movement of the mouse is tracked by a camera located above the maze using software from Noldus (Ethovision). The output data is a representation of the number of times the animal has entered into the open and closed arms.

3.9.3 Open Field

This is a test that measures fear, anxiety and motor function based upon an animal's desire to explore a novel environment and its fear of exploring a brightly lit open area. The mouse is placed in the upper right corner of each open field box. The square box is 45cm wide and 45cm high. After the mouse is placed in the box, it has ten minutes to freely explore all areas of the box. The behavior of the mouse is recorded by a video camera located above the box connected to the ceiling. The data output is comprised of the amount of time the mouse has spent in the center of the box in comparison to the corners. The video tracking software used for open field testing is Noldus (Ethovision).

3.9.4 Fear Conditioning

The fear conditioning test is a standard test for learning and memory. Learning is measured in the form where fear is associated with a neutral stimulus (tone) which is paired with an aversive stimulus (shock). After teaching the mice the association between the tone and shock, the neutral stimulus alone should be able to elicit fear. To measure fear, the mouse's freezing reaction is recorded when put into a sudden fearful situation. This test takes three days. The first day is dedicated to "training" the mice. In brief, the mouse is placed in the fear conditioning cage for six minutes in which they are free to explore. After two minutes, a tone is played from within the apparatus for 30 seconds, ending with a 2 second 0.5 milliamp shock. One minute after the shock the tone is repeated for 30 seconds followed by another 0.5 milliamp shock for 2 seconds. In the last two minutes, there is no tone or shock. Over the duration of the six minutes, the freezing behavior of the mouse is recorded. The second day of testing is contextual conditioned fear testing and must be done 24 hours after day one. The mouse is placed in the same apparatus with identical lighting and room conditions as day one. The mouse's freezing behavior is recorded for six minutes and measures the fear associated with being placed back into the same environment where an adverse stimulus (shock) was delivered. On the third day of testing, the mouse is placed in a novel environment with a novel smell and light. Once the mouse is placed in the novel environment, its freezing is recorded for three minutes to ensure the animals do not associate the novel environment from the environment on day one and two. After three minutes, the tone that was played on day one is played for 30 seconds and the freezing is recorded, measuring the fear associated with the tone.

4 RESULTS

4.1 Morphological Analysis of Mutant Mice with the I56i, vI56i-I12b and I56i-I12b *Dlx* Enhancer Deletion(s).

In characterizing the viability and fertility of ΔI56i mutant mice, I mated mice heterozygous for the I56i enhancer deletion to generate homozygous ΔI56i mice. The offspring from the heterozygous mice were screened for the absence of the I56i enhancer. Homozygous mutants did not show any morphological abnormalities or size differences when compared to their wild-type siblings (results based on ~30 homozygous ΔI56i mutant mice). Once ΔI56i mutants were generated I allowed them to mate with one another to determine if the absence of the I56i enhancer had an effect on the mouse's ability to breed. The mutant mice had no issues breeding and produced normal size litters. In order to investigate any size or morphological changes in ΔI56i mutant embryos I carried out over 20 mouse mating's in which the males and females were both I56i heterozygous mutants. I collected embryos at E11.5, E14.5 and P0 and none had noticeable morphological or size differences with respect to their bodies and brain when compared to their wildtype siblings (Fig 4.1). -

In an attempt to assess the functional consequence of mice that harbor two *Dlx* enhancer deletions, I used a second mouse line that had a SNP of an adenine for a guanine in the center of the ΔI56i enhancer and that lacked the ΔI12b enhancer (Pelletier 2011). Previous Ekker lab members have individually characterized the consequences in mice of having a SNP in the I56i enhancer and lacking the I12b enhancer. I selectively mated mutant I12b mice with "knock-in" vI56i mice to obtain homozygous vI56i-I12b mice. These mice are both viable, fertile and are indistinguishable with respect to their body size and morphology compared to the wild type controls and this is based on the birth of over 20

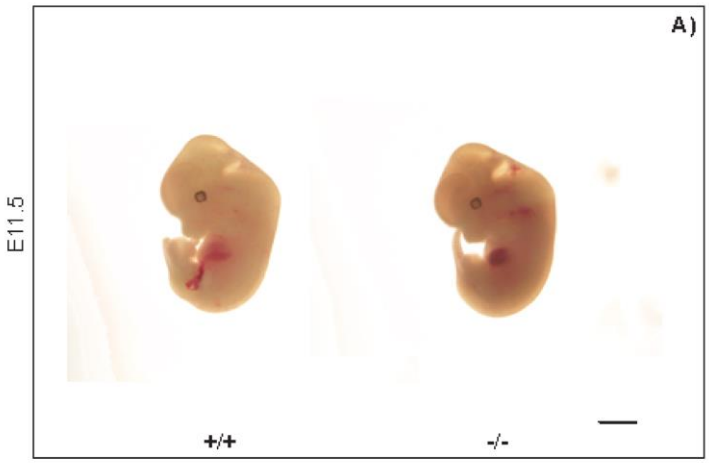
vI56i-I12b mice (Figure 4.2).

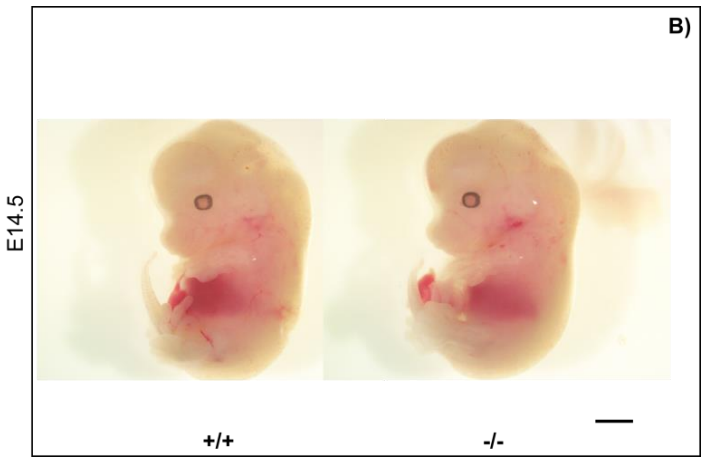
The third mouse line I generated lacked both the I56i and I12b enhancer. To generate mice homozygous for both enhancer deletions I mated a homozygous I56i mouse with a heterozygous I12b mouse (Yu 2011). Double homozygous mice were infrequently born and litter sizes were much smaller than the average wildtype litter. It is possible that the low frequency of homozygous I56i-I12b mice births is due to the embryos not surviving past a certain developmental stage and being reabsorbed into the mother. In the rare cases where homozygous I56i-I12b embryos do survive until term, they do not live past post natal day 5 and exhibit a dwarfed body size. These mice look weak and seem to have limited motor ability. Female mice that are heterozygous for both the I56i and I12b deletion seem to have difficulty becoming pregnant and when they do, the litter is half the size of a standard wild type litter. These results are based off of 8 heterozygous I56i-I12b pairings that produced 4 homozygous I56i-I12b births.

4.2 Quantitative PCR Assays and *Dlx* Gene Expression.

In characterizing the consequence of the various enhancer deletion(s) on *Dlx* function I performed qPCR assays on cDNA that was synthesized from RNA extracted from the ventral telencephalon of mutant and wildtype mice at three developmental stages; E11.5, E14.5 and P0. In all cases, expression in mutants was compared to wildtype siblings and gene expression level were analyzed using six biological replicates each assayed in experimental triplicates. All four *Dlx* genes expressed in the forebrain were characterized along with *Gad* gene transcripts, which may be under partial DLX5/DLX6 control. The non-coding RNA, *Evf*-2 was also assayed because of its ability to form a stable complex with

Figure 4.1: Morphological analysis of ΔI56i mutants at various developmental stages. A-B) Sagittal view of E11.5 and E14.5 ΔI56i mutant embryos. There is no noticeable difference in size or morphology between the ΔI56i mutant and wildtype embryos. **C)** Dorsal view of dissected P0 ΔI56i mutant and wildtype brains. There is no obvious change in P0 ΔI56i mutant brains compared to the wildtype control. **Scale bar**: 1000µm.


A)
E11.5
+/+
-/-


B)
E14.5
+/+
-/-

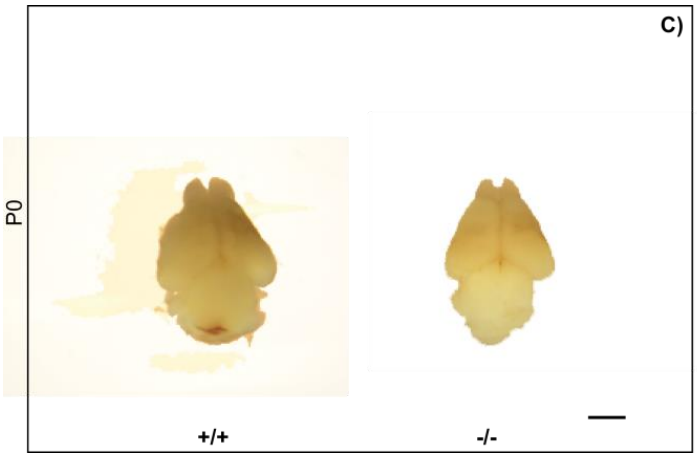
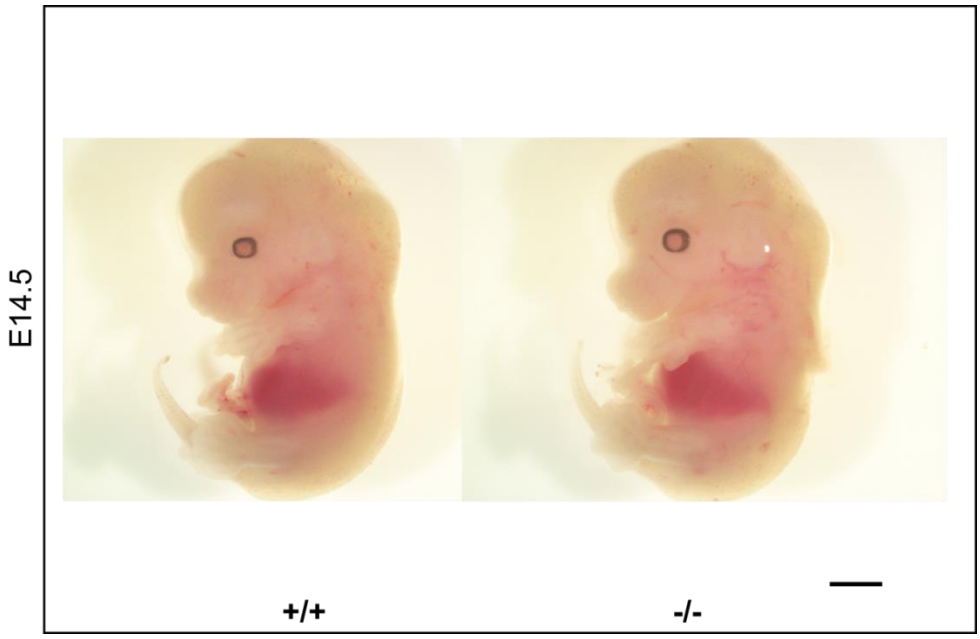

C)
P0
+/+
-/-

Figure 4.2 Morphological analyses of vI56i-I12b mutants at E14.5. Sagittal view of E14.5 vI56i-I12b mutant embryos. There is no noticeable difference in size or morphology between mutant and wildtype embryos. **Scale bar:** 1000µm.

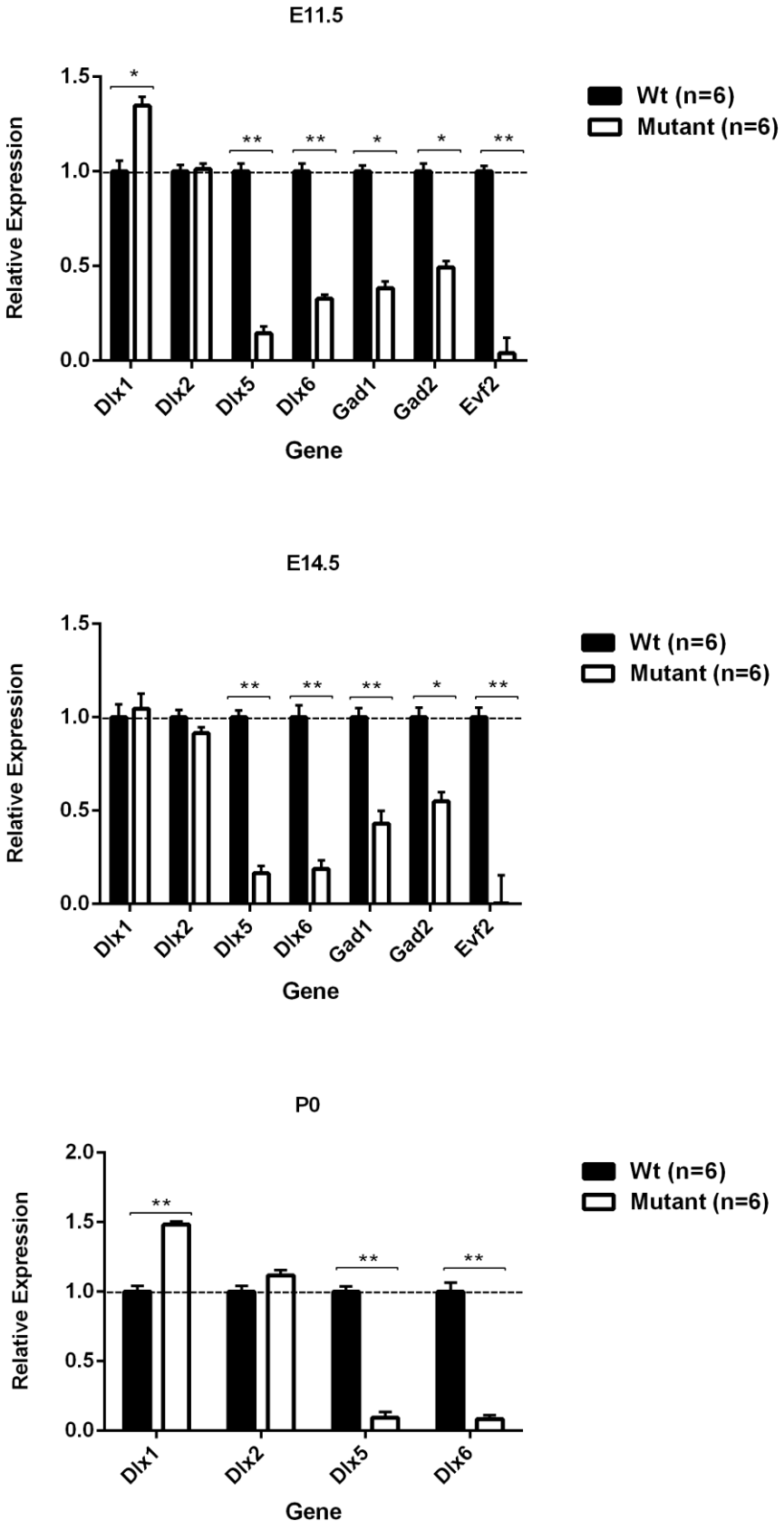

E14.5
+/+
-/-

DLX2, playing a role in regulating transcription of *Dlx5*/*Dlx6* (Feng et al. 2006). Individual primers were designed and optimized according to the *Minimum Information for Publication of Quantitative Real-Time PCR Experiments* (MIQE Guidelines) and data were analyzed using the Pfaffl method of analyzing qPCR data. The "housekeeping" gene *elongation factor 1-alpha (ef1-α)* was used to standardize all results. Calculations are presented in relative units as the mean ± SEM.

4.2.1 The Absence of the I56i Enhancer Significantly Decreases *Dlx5/6, Gad* and *Evf-2.*

In ΔI56i mutant mice, there is a significant increase in *Dlx1* transcript (~40%) and no significant change in *Dlx2* transcript levels at any of the three time points tested; E11.5, E14.5 and P0. However, the relative expression of *Dlx5* and *Dlx6* drastically decreases (~80%), indicating the importance of I56i in regulating the transcription of these genes. Similarly, *Gad1* and *Gad2* which are essential in producing enzymes that synthesize the inhibitory neurotransmitter GABA are significantly decreased (~60%), suggesting these mice may have delayed or disrupted GABAergic neuron development. Lastly, the non-coding RNA, *Evf*-2 significantly decreases by (~90%) (Fig. 4.3).

Figure 4.3: ΔI56i mutants show impaired *Dlx, Gad* and *Evf-2* expression in the forebrain. Quantitative Real Time qRT-PCR analysis displaying relative expression levels of *Dlx1/Dlx2/Dlx5/Dlx6, Gad* and *Evf-2* transcripts in the mouse ventral telencephalon of E11.5 I56i mutants. Relative gene expression levels of I56i mutant mice were normalized to transcript levels found in the ventral telencephalon of wildtype mice. The error associated with each data point is a statistical representation of the standard error of the mean for each gene relative to the normalization control (*Ef1α*) in mice. Experiments were performed in triplicates and statistical significance of data was analyzed using a Two tailed T-test (α=0.05). **P*<0.05, ***P*<0.02.


E11.5
Relative Expression
1.5
1.0
0.5
0.0
*
**
**
*
*
**
Dlx1
Dlx2
Dlx5
Dlx6
Gad1
Gad2
Evf2
Gene
Wt (n=6)
Mutant (n=6)
E14.5
Relative Expression
1.5
1.0
0.5
0.0
**
**
**
*
**
Dlx1
Dlx2
Dlx5
Dlx6
Gad1
Gad2
Evf2
Gene
Wt (n=6)
Mutant (n=6)
P0
Relative Expression
2.0
1.5
1.0
0.5
0.0
**
**
**
Dlx1
Dlx2
Dlx5
Dlx6
Gene
Wt (n=6)
Mutant (n=6)

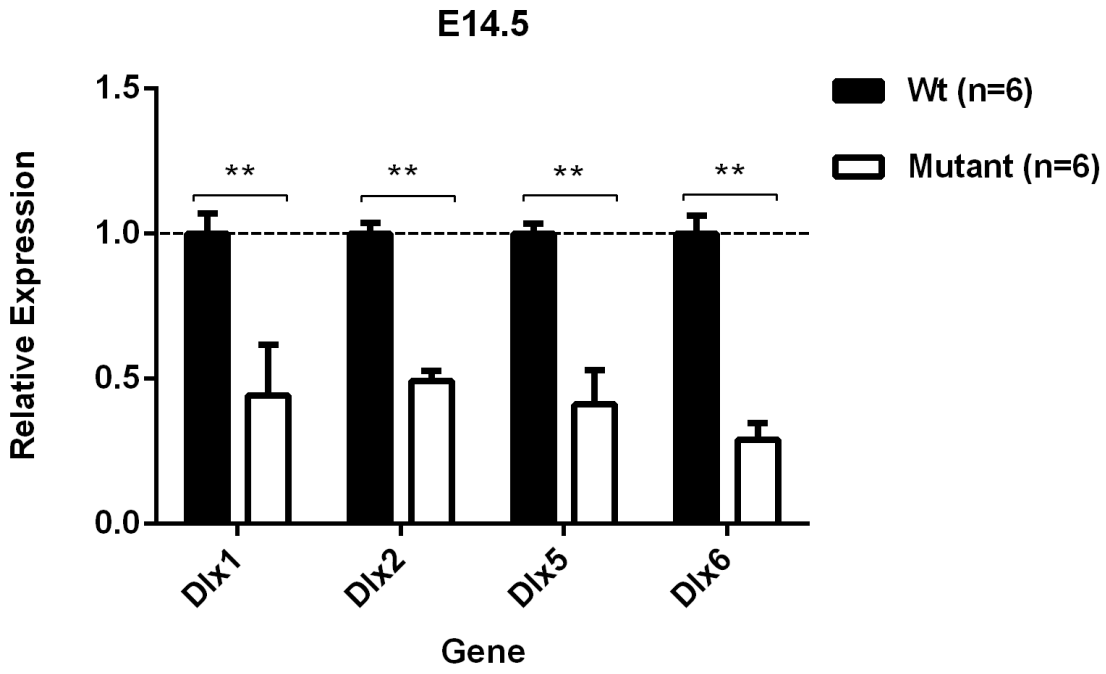
4.2.2 An SNP in the I56i Enhancer in Combination with the I12b Enhancer Deletion Significantly Impairs *Dlx* Gene Expression.

I performed qPCR assays on mice homozygous for both the vI56i and I12b mutations at E14.5 to determine the combined effect of two enhancer mutations on *Dlx* expression. The results have shown that at E14.5, *Dlx1/Dlx2/Dlx5* and *Dlx6* expression all significantly decrease by approximately 50% for all genes. It seems like the decrease in *Dlx* expression may be the result of the additive effects of the mutations on relative gene expression levels that were previously observed in mice that harbored a SNP in the I56i enhancer and mice that lacked the I12b enhancer (Fig. 4.4).

4.3 *In situ* Hybridization Confirms qPCR Data and Shows a Global Decrease of *Dlx5/6,Gad* and *Evf-2* Expression in the Ventral Telencephalon of ΔI56i Mutant Mice.

I performed *in situ* hybridization on mutant forebrain tissue to qualitatively see changes in *Dlx* expression patterns and confirm that the absence of the I56i enhancer is related to loss of *Dlx*, *Gad* and *Evf-2* expression. Antisense RNA probes for *Dlx1/Dlx2/Dlx5/Dlx6, Gad* and *Evf*-2 were used on E11.5 and E14.5 ΔI56i mutant mouse ventral telencephalon tissue sections. Eisenstat *et al*. have shown *Dlx1* and *Dlx2* to be expressed in the immature cells of the VZ and SVZ of the MGE and LGE and *Dlx5/Dlx6* to be expressed in the SVZ and MZ (Eisenstat et al. 1999) which is consistent with my results. The expression patterns remain stable between mutant and wildtype mice at both E11.5 and E14.5 (Fig. 4.5-4.6). However, the expression signals in the ΔI56i mutants show a slight global increase in *Dlx1* expression in the VZ and SVZ at E11.5 and E14.5, while no changes in expression level or pattern are seen with *Dlx2*. The expression level of *Dlx5, Dlx6, Evf-2*

Figure 4.4: vI56i-I12b mutants show impaired *Dlx* expression in the forebrain. Quantitative Real Time-PCR analysis displaying relative expression levels of *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* transcripts in the mouse ventral telencephalon of E14.5 vI56i-I12b mutants. Relative gene expression levels of vI56i-I12b mutant mice were normalized to transcript levels found in the ventral telencephalon of wildtype mice. The error associated with each data point is a statistical representation of the standard error of the mean for each gene relative to the normalization control (*Ef1α*) in mice. Experiments were performed in triplicates and statistical significance of data was analyzed using a Two tailed T-test (α=0.05). **P*<0.05, ***P*<0.02.


E14.5
Wt (n=6)
Mutant (n=6)
Relative Expression
1.5
1.0
0.5
0.0
**
**
**
**
Dlx1
Dlx2
Dlx5
Dlx6
Gene

and *Gad* are significantly decreased in the SVZ and MZ, demonstrating the effect the absence of the I56i enhancer has on *Dlx* forebrain expression (Fig. 4.5-4.6).

4.4 Immunohistochemistry Reveals Changes in a Population of GABAergic Neuronal Markers in Forebrain.

In determining the role of the I56i enhancer in neuronal differentiation, we performed immunohistochemistry experiments on the somatosensory cortex of P35 ΔI56i mutant and wildtype mice. We stained the forebrains with antibodies for calbindin, parvalbumin and GABA (Fig. 4.7-4.9). Calbinding and parvalbumin are calcium binding proteins that are representative of subpopulations of GABAergic neurons and GABA is the chief inhibitory transmitter used by GABAergic neurons in the forebrain. We qualitatively observed that the number of cells stained for either calbindin or parvalbumin in the mutant and wildtype somatosensory cortex of mutant ΔI56i mice compared to the wildtype. Although a quantitative analysis was not performed there is a noticeable decrease of $GABA^+$ cells in the ΔI56i mutant brain.

4.5 Δ I56i Mutant Mouse Behavior

Dysfunction in the *Dlx* genes has been associated with a variety of neurological disorders such as autism spectrum disorder, schizophrenia and anxiety (Poitras 2010). Since the ΔI56i enhancer is involved in regulating at least some of the *Dlx* genes, I performed a series of four behavioral tests to see if the mice lacking this enhancer have a behavioral phenotype. Behavior testing was performed at the Behavioral Core Facility at the Roger Guidon Hall at the Univeristy of Ottawa. The ΔI56i mutant and wildtype control mice were bred at the Vanier Mouse Facility at the University of Ottawa. Once the pups reached three weeks of age

Figure 4.5: *In situ* hybridization on coronal sections of E11.5 embryonic ventral telencephalon forebrain tissue from wildtype (A-F) and ΔI56i mutants (A′-F′). In the ΔI56i mutants, *Dlx1* expression increases **(A-A′)** and no change is seen in *Dlx2* **(B-B′)**. The ΔI56i mutant mice show a significant global decrease of *Dlx5* **(C-C′)**, *Dlx6* **(D-D′)**, *Gad2* **(E-E′)** and *Evf*-2 **(F-F′)** mRNA expression patterns in the VZ and SVZ of the ventral telencephalon. **Scale bar:** 50μm.

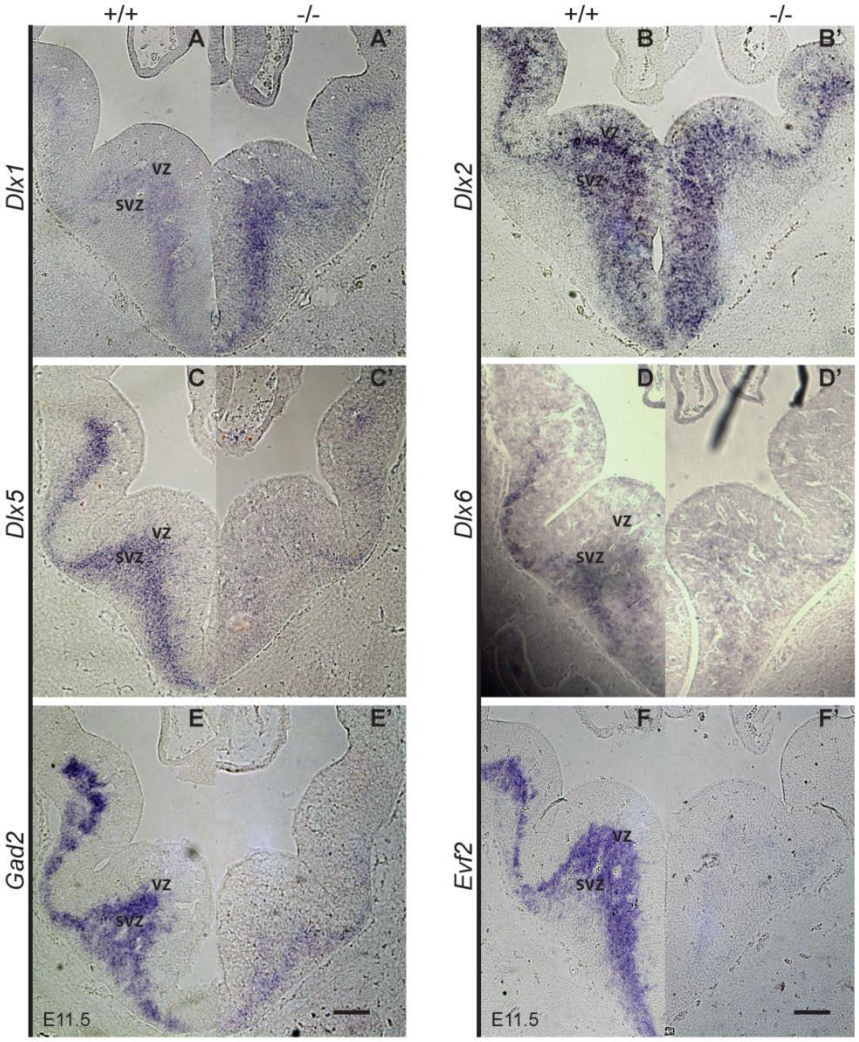

+/+
-/-
+/+
-/-
A
A'
B
B'
C
C'
D
D'
E
E'
F
F'
Dlx1
Dlx2
Dlx5
Dlx6
Gad2
Evf2
VZ
SVZ
E11.5
E11.5

Figure 4.6: *In situ* hybridization on coronal sections of E14.5 embryonic ventral telencephalon forebrain tissue from wildtype (A-E) and ΔI56i mutants (A′-E′). In the ΔI56i mutants, *Dlx1* expression increases **(A-A′)** and no change is seen in *Dlx2* **(B-B′)**. The ΔI56i mutant mice show a significant global decrease of *Dlx5* **(C-C′)**, *Dlx6* **(D-D′)**, *Evf*-2 **(E-E′)** mRNA expression patterns in the VZ and SVZ of the ventral telencephalon. **Scale bar:** 50μm.

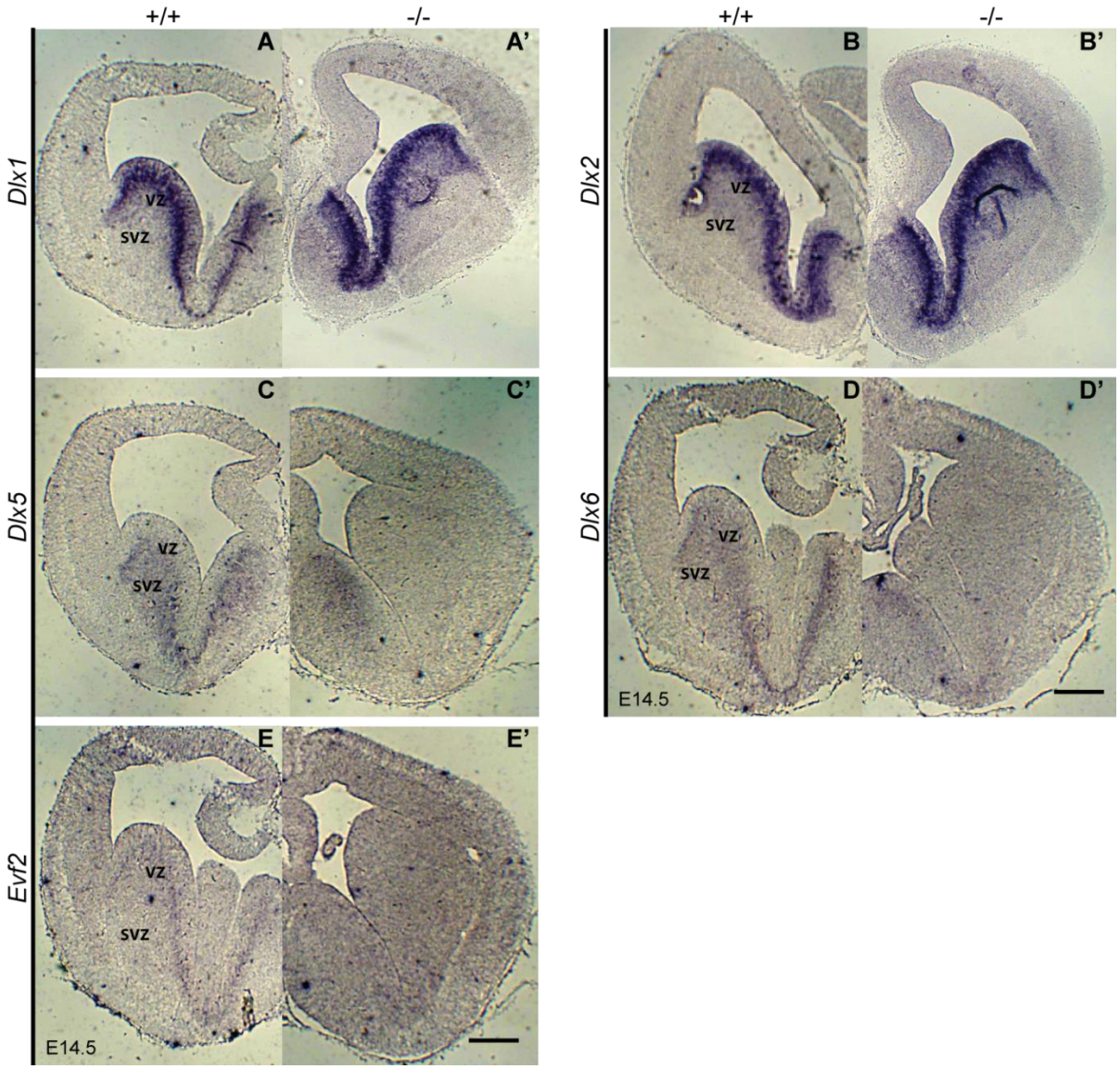

+/+
-/-
+/+
-/-
A
A'
B
B'
C
C'
D
D'
E
E'
Dlx1
Dlx2
Dlx5
Dlx6
Evf2
VZ
SVZ
VZ
SVZ
VZ
SVZ
VZ
SVZ
VZ
SVZ
E14.5
E14.5

Figure 4.7: Calbindin expressing cells in wildtype and ΔI56i mutant mouse somatosensory cortex at P35. Cells of the somatosensory cortex immunostained with an antibody against calbindin at 10x magnification in ΔI56i mutants (B) are generally comparable to that detected in the wildtype controls (A). (A'-B') represent an increased magnification of an area of A) and B) highlighted with a white box at 20x magnification.

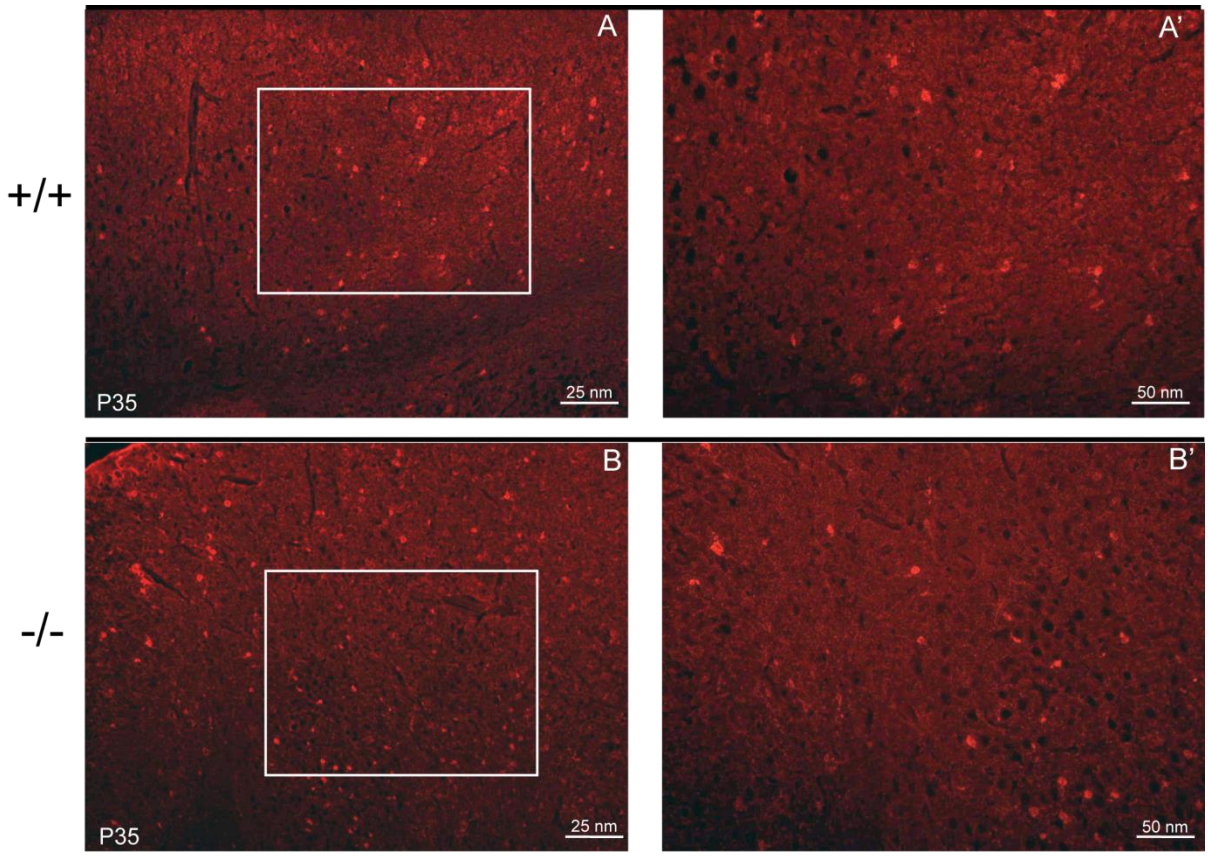

+/+
A
A'
P35
25 nm
50 nm
-/-
B
B'
P35
25 nm
50 nm

Figure 4.8: Parvalbumin expressing cells in wildtype and ΔI56i mutant mouse somatosensory cortex at P35. Cells of the somatosensory cortex immunostained with an antibody against parvalbumin at 10x magnification in ΔI56i mutants (B) are generally comparable to that detected in the wildtype controls (A). (A'-B') represent an increased magnification of an area of A) and B) highlighted with a white box at 20x magnification.

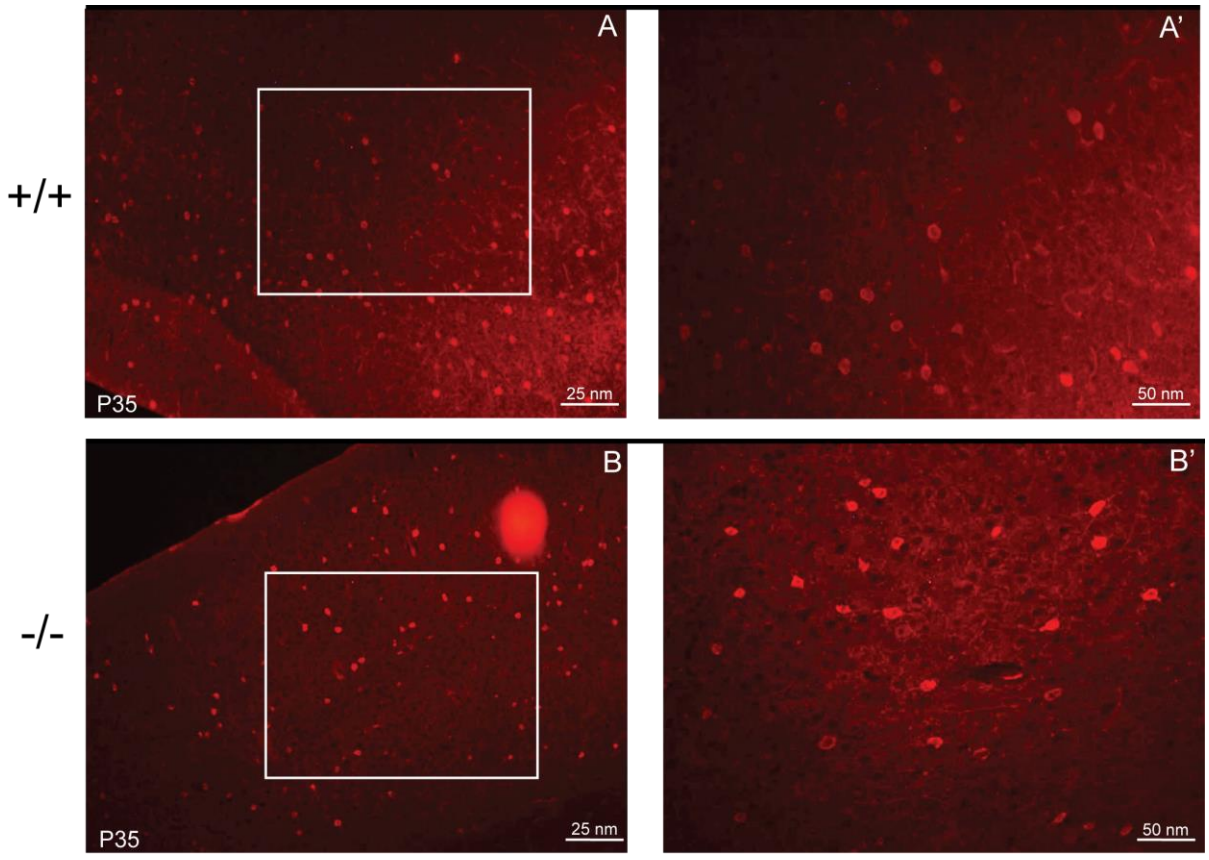
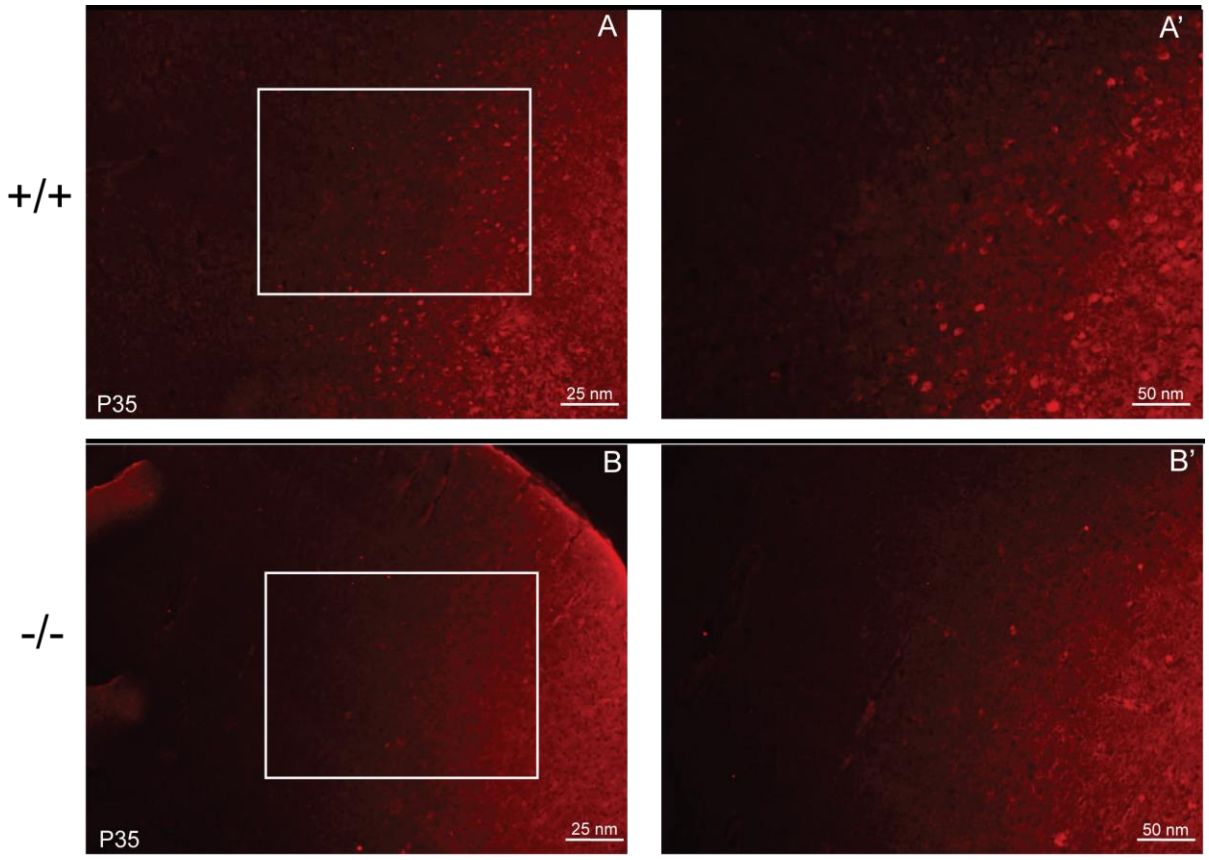

+/+
-/-
A
A'
B
B'
P35
P35
25 nm
25 nm
50 nm
50 nm

Figure 4.9: GABA expressing cells in wildtype and ΔI56i mutant mouse somatosensory cortex at P35. Cells of the somatosensory cortex immunostained with an antibody against GABA at 10x magnification in ΔI56i mutants (B) seem to have a decreased number of stained cells compared to that detected in the wildtype controls (A). (A'-B') represent an increased magnification of an area of A) and B) highlighted with a white box at 20x magnification.


+/+
-/-
A
A'
B
B'
P35
P35
25 nm
25 nm
50 nm
50 nm

I tagged their ears and clipped their tails for genotyping. At three weeks of age the mice were transferred to the Roger Guidon Mouse Facility and housed in quarantine for four weeks to acclimate to the new environment. Twenty ΔI56i mutant mice were tested and compared to twenty wildtype littermate controls, each genotype having an equal number of males and females. Sample number represented in each figure represents the number of mice included in the final statistical calculation, not including outliers. No significant differences were found within sexes and the data presented in this report only takes into account differences found between genotypes.

The first behavior test I performed was beam break, which is used to distinguish any differences in general locomotor activity. In this test the mice were placed in a cage and allowed to explore for two hours. During this time the number of beam breaks of the mice is recorded. During the first hour of testing, the mice are acclimating to a new environment and the second hour is used to identify any gross differences in motor function. The average activity of the ΔI56i mutants and wildtype mice were calculated using a T-test and no significant locomotor differences were identified T test, $T(31.05) = -1.955$, $p < 0.06$ (Fig. 4.10). There is a trend towards significance and it would be of interest to repeat this test for a longer duration, leaving the mice to explore over night when they are most active.

The day following the beam break test I subjected the mice to the elevated plus maze to measure differences in fear and anxiety. The premise of this test is based on the preference of the animal to explore dark enclosed spaces compared to bright exposed places. The mice were placed in the maze and free to explore for ten minutes. During this time a camera traced and recorded their path. Both the wildtype and mutant mice spent more time in the closed arms compared to the open arms. One-way ANOVA revealed that the percent time spent in the open arms of the maze was not significantly different between the mutant and wildtype

mice, but there seems to be a trend towards significance ($p < 0.077$) (Fig. 4.11, A). Furthermore, a one-way ANOVA revealed that the percent time spent in closed arms was not significantly different between the mutant and wild type mice (Fig.4.11, B). These results lean towards a suggestion that mutant ΔI56i mice might be slightly more anxious than the wildtype mice since there is a trend towards significance in the number of times the mutant mice enter the open area compared to wildtype. It would be of interest to repeat this test with a larger sample size to see if changes reach statistical significance.

Since defects within the *Dlx* genes have been associated with anxiety disorders I decided to subject the ΔI56i mutant mice to an open field test. This test measures fear, anxiety and motor function based upon the desire of the mouse to explore a novel environment and its fear of exploring a brightly lit open area. I began the test by placing a mouse in the upper right hand corner of an open white box, allowing the mouse to explore for ten minutes while its behavior was recorded. The data output is a representation of the

Figure 4.10: Beam Break. The average Locomotor activity is shown for wild type and ΔI56i mutant mice. A trend towards increasing activity is observed for the mutant mice compared to the wild type controls. T test, $T(31.05) = -1.955$, $p < 0.06$.

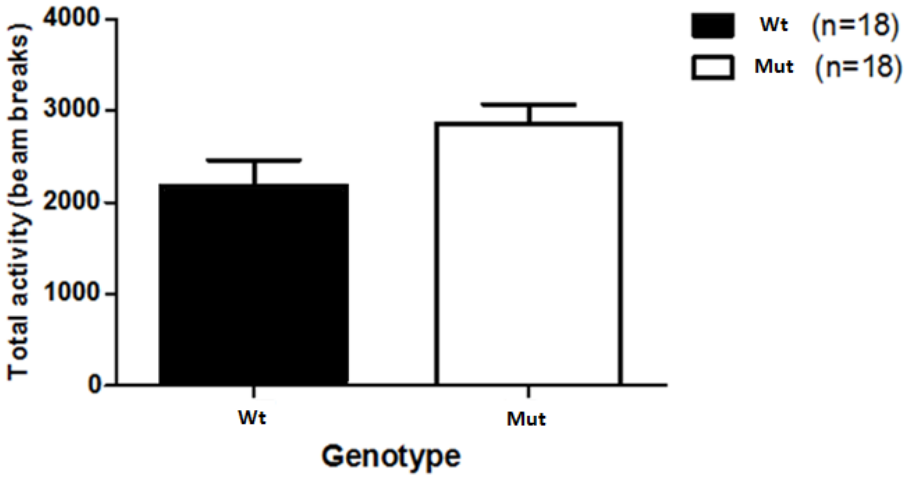
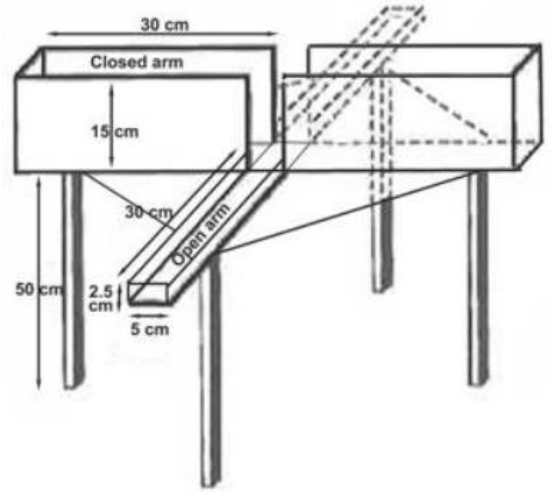
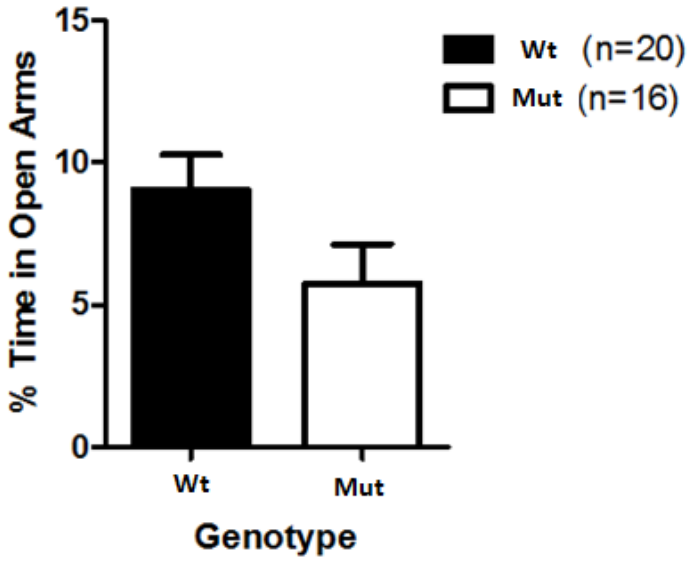

Wt (n=18)
Mut (n=18)
Total activity (beam breaks)
4000
3000
2000
1000
0
Wt
Mut
Genotype

Figure 4.11: Elevated plus maze. The percentage of time spent in the open and closed arms of an elevated plus apparatus is shown for wild type and ΔI56i mutant mice. **A)** One-way ANOVA revealed that the percent time spent in open arms was not significantly different between the mutant and wild type mice. However, there is a trend towards significance ($p < 0.077$). **B)** One-way ANOVA revealed that the percent time spent in closed arms was not significantly different between the mutant and wild type mice.

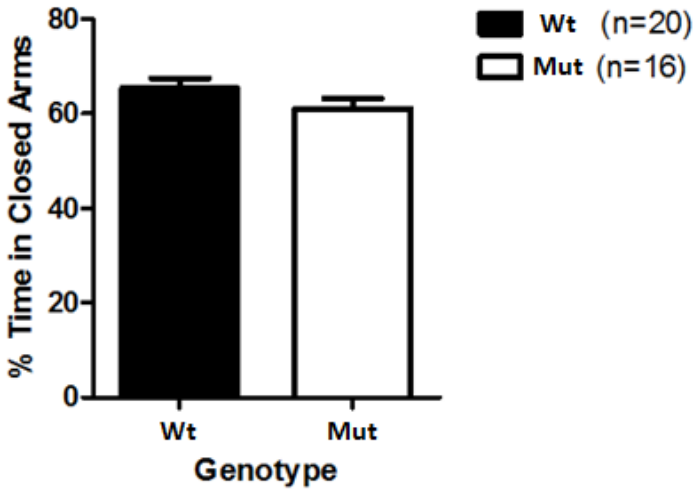
A)


30 cm
Closed arm
15 cm
30 cm
Open arm
50 cm
2.5 cm
5 cm

B)


% Time in Open Arms
15
10
5
0
Wt
Mut
Genotype
Wt (n=20)
Mut (n=16)

C)

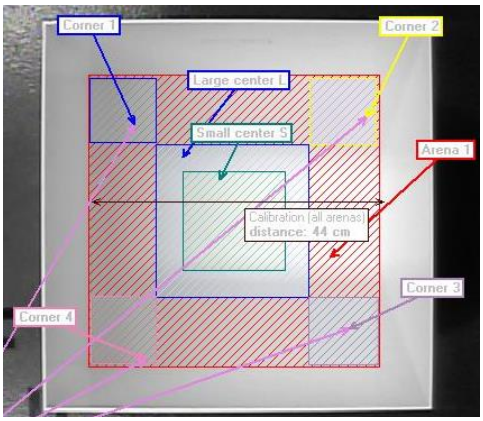

% Time in Closed Arms
80
60
40
20
0
Wt
Mut
Genotype
Wt (n=20)
Mut (n=16)

amount of time each mouse spends in a specific region or arena of the open box over ten minutes (Fig 4.12, A). The output data were analyzed using a T-test and results concluded that the ΔI56i mutant mice spend a significantly less amount of time in the small center compared to the wildtype controls, T test: T (26.4) = -2.2, p = 0.035 (Fig.4.12, A-B). There was no difference seen in the amount of time the wildtype and mutant mice spent in the large center (Fig.4.12, A, C). T test: T (30.9) = -1.98, p < 0.057. The results from this test may provide insights into a possibility that the absence of the I56i enhancer may be related to an increased level of fear and anxiety.

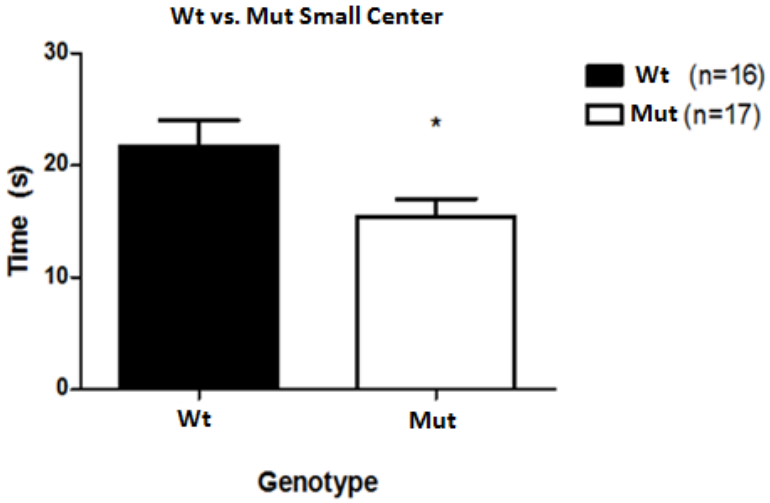
The last behavior test I performed was fear conditioning which measures learning and memory. Since the *Dlx* genes have been related to certain symptoms associated with neurological disorders, subjecting the mutant mice to fear conditioning has allowed me to evaluate if the absence of the I56i enhancer contributes to memory loss or learning disabilities. The first day of testing is baseline training in which the mice are "trained" to associate a neutral stimulus (tone) with an aversive stimulus (shock). Baseline training entails placing the mice into individual cages for six minutes. After two minutes a tone is played for 30 seconds ending with a 0.5 milliamp shock. One minute following the shock, the original tone is repeated for another 30 seconds followed by the same 0.5 milliamp shock for two seconds. During the last two minutes of the test, there is no tone or shock. During the entirety of the test, the freezing behavior of the mouse is recorded. The results from the first day show that baseline training was successful in that all wildtype and ΔI56i mutant mice showed very limited freezing activity, indicating that they did not associate the tone with an aversive stimulus, the shock. It is important the mice did not show extensive freezing once they were exposed to the tone, otherwise this stimulus would not be sufficient in indicating the mouse's ability to learn and associate the tone with the shock in subsequent days of

Figure 4.12: Open Field. The amount of time spent in the large and small center of an open field apparatus is shown for wild type and ΔI56i mutant mice. **A)** T-test showed that there is a significant difference in the time spent in the small center between the mutant and wild type mice. T test: T (26.4) = -2.2, p = 0.035. **B)** T test demonstrates that there is no significant difference in the time spent in the large center between mutant and wild type mice. However, there is a trend towards significance and a larger sample size might show that mutant mice spend more time in the center of the open field. T test: T (30.9) = -1.98, p < 0.057.

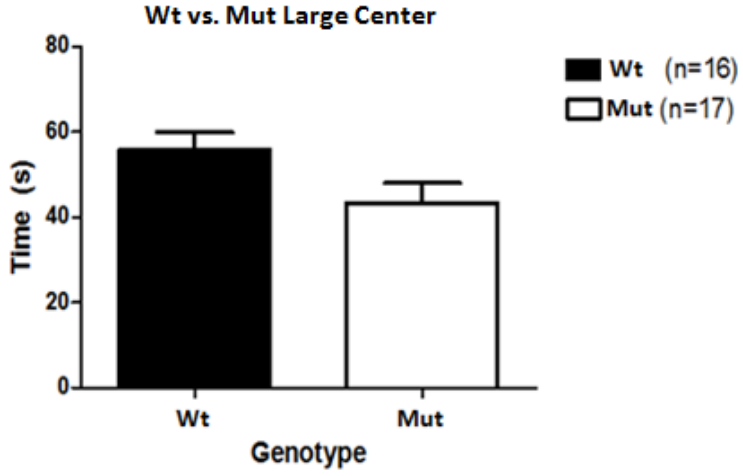
A)


Corner 1
Corner 2
Large center 1
Small center 5
Arena 1
Calibration (all arenas)
distance: 44 cm
Corner 3
Corner 4

B)

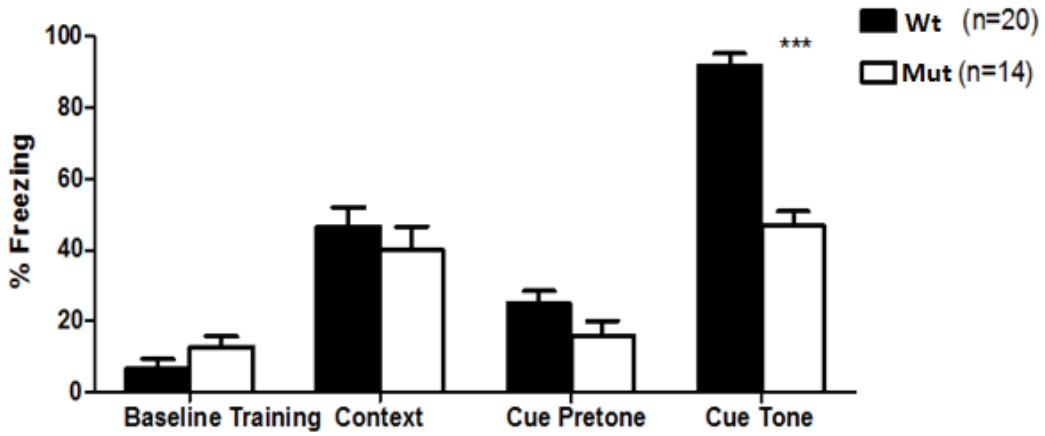

Wt vs. Mut Small Center
Wt (n=16)
Mut (n=17)
Time (s)
30
20
10
0
*
Wt
Mut
Genotype

C)


Wt vs. Mut Large Center
Wt (n=16)
Mut (n=17)
Time (s)
80
60
40
20
0
Wt
Mut
Genotype

testing (Fig.4.13). The second day was dedicated to contextual training which involved placing the mice in the same apparatus as day 1 with all identical conditions. Figure 4.13 "context" shows that both the wildtype and mutant mice had similar freezing behavior, demonstrating that the mice remembered and were aware that the environment they were placed in was associated with an aversive stimulus from the previous day. Context dependent fear conditioning is associated with hippocampal function, indicating that mutants do not have defects in their short-term memory. On the third day of testing the mice were placed in a novel environment and subjected to an identical tone as in day 1 for three minutes after three minutes of silence. The freezing behavior of the mice was recorded. The "cue pretone" in Figure 4.13 is a representation of the average freezing behavior of the mice during the first three minutes of the test before any tone was administered. The minimal freezing behavior of the wildtype and mutant mice is an indication that the mice did not associate the novel environment with a foot shock. Following the three minutes of silence or pretone, the mice were subjected to a 3 minute tone. The results in Fig 4.13 "cue tone" show a significant difference in the freezing behavior of the ΔI56i mutants and wildtype mice. Once the tone is initiated in a novel environment, the mutant mice showed limited freezing in comparison to their wildtype counterparts, indicating the mutants did not learn that the neutral tone was associated with an aversive stimulus, the shock. On average, the wildtype mice had a very high level of freezing each time they heard the tone, demonstrating their ability to associate the tone with a shock. Overall, these results suggest that the ΔI56i mutant mice have a functioning memory and were able to recognize during "context" that the environment they were placed in was associated with an aversive stimulus from the day before. On the third day of testing, the mutants were unable to associate the tone with the shock and had significantly lower freezing in comparison to the wildtype mice. Inability to associate the

Figure 4.13: Fear Conditioning. Repeated ANOVA showed no significant difference in freezing between ΔI56i mutants and wild type mice during either baseline training or context. During cue tone, the mutant mice had a significantly lower level of freezing compared with the wild type controls. Overall, there is an effect of the testing day ($F_{(3,96)=}105.6$, $p<0.001$) and of day*genotype ($F_{(3,96)=}18.1$, $p<0.001$).


Wt (n=20)
Mut (n=14)
***
% Freezing
100
80
60
40
20
0
Baseline Training
Context
Cue Pretone
Cue Tone

the tone with a foot shock may suggest the mutants have defects within their amygdala.

5 Discussion

5.1 The I12b and I56i Enhancers Demonstrate Functional Redundancy in the Developing Mouse.

The spatial and temporal expression of *Dlx* genes is tightly regulated by a class of *cis*-regulatory elements called enhancers. These elements respond to various cues, binding to transcription factors to stimulate transcription, establishing patterns of gene expression in diverse tissues at specific times (Wolpert and Tickle 2011). Enhancers involved in regulating developmental genes are distinguished through their pronounced level of sequence conservation throughout species, indicating their functional importance in regulating growth and survival during development (Pennacchio et al. 2006). The *Dlx cis*-regulatory elements are highly conserved throughout evolution and show very little sequence variation among vertebrate species (Ghanem et al. 2008). Based on this theory, any mutation or absence of a highly conserved element should dramatically disrupt the morphology, growth or survival of the individual. Such an example is demonstrated in mice with an absent conserved element, I37-2 located within the intergenic region of the *Dlx3/Dlx4* loci. These individuals have morphological defects and fail to produce visceral arches (Sumiyama and Ruddle 2002).

In characterizing the functional importance of the I56i enhancer on development I analyzed the morphology, viability and fertility of ΔI56i mutant mice. These mice appear normal and do not have morphological abnormalities or size differences of the body or brain. The ΔI56i mutant mice are phenotypically indistinguishable from their wildtype siblings and have no issues breeding and all litters are of standard size. These findings implicate that the

I56i enhancer is not essential for the overall well-being, growth and survival of mice. Similarly, mice harboring an I12b enhancer deletion develop normally and are phenotypically indistinguishable from both heterozygous and wildtype mice littermates (Yu 2011). The seemingly normal appearance of mice with a single I56i or I12b deletion contradict the theory suggesting that the high level of conservation found within the *Dlx* enhancers is a result of their importance during early development. The absence of a morphological phenotype found in the mutants may be due to functional redundancy between I56i and I12b. Both the I56i and I12b enhancers have overlapping domains of activity with two putative DLX binding sites that each harbor a putative homeodomain binding site (TAAT), suggesting functional redundancy and cross-regulation (Ghanem et al. 2008; Poitras and Ghanem et al. 2007). Functional redundancy is also seen within the *Dlx* genes themselves due to overlapping patterns of expression. Single *Dlx* mutants are morphologically normal and compensate each other's loss of function, while double *Dlx* mutants from the same bigene pair have severe abnormal morphological phenotypes (Panganiban and Rubenstein 2002).

In testing the above hypothesis, two different mice lines harboring multiple deletions were generated. The first line completely lacks the I12b and I56i enhancers (ΔI12b-ΔI56i) and the second lacks the I12b enhancer and has a SNP of an adenine for a guanine in a *Dlx* binding motif of I56i (ΔI12b-vI56i). Mice that harbor the single SNP mutation in the I56i enhancer do not have noticeable morphological changes and their growth and survival are not compromised (Pelletier 2011). As previously mentioned, the high conservation of enhancers throughout species suggests their importance in development and any mutation in their binding sequences may be detrimental to the survival of the individual. There are countless studies demonstrating the negative effects of SNP mutations in conserved

sequences causing unfavorable morphological changes. One such example is seen in mice that harbor a SNP in a *cis*-regulatory element that governs *Shh* expression. The presence of this SNP causes preaxial polydactyly syndrome in mice that are left with extra digits (Lettice et al. 2002).

Mice that are homozygous for both ΔI12b-vI56i mutations are viable, fertile and do not demonstrate noticeable phenotypic abnormalities, rendering them indistinguishable from their wildtype siblings. This is surprising since the SNP is located in an ultraconserved *Dlx* binding motif which should inhibit the ability of the I56i enhancer to functionally compensate for the loss of I12b. In vI56i mice, the activity of I56i significantly decreases and competitive EMSA experiments have shown that *Dlx* proteins have a decreased affinity for the *Dlx* binding motif harboring the SNP (Pelletier 2011). However, the SNP is located in only one of two *Dlx* binding domains in I56i and we can assume that the binding of DLX proteins to the second *Dlx* binding domain is not disrupted. The presence of a SNP in one of two binding sites is not significant in affecting the ability of the I56i enhancer to compensate for the loss of I12b function.

In generating mice that harbor a double enhancer deletion of I12b and I56i, I have confirmed that the two *Dlx* enhancers have partial functional redundancy. In contrast to the ΔI12b-vI56i mouse line, ΔI56i-ΔI12b mice were infrequently born and litter sizes were much smaller than the average wildtype litter. In the unique case in which homozygous ΔI56i-ΔI12b embryos survive until term, they do not live past post natal day 5 and exhibit a dwarfed body size. These mice look weak and seem to have limited motor ability. The phenotypic changes that occur in these mice correspond to the idea that when one of two *Dlx* enhancers is deleted and or mutated; the remaining functional enhancer has the ability to compensate for the others absence or decrease in activity, promoting survival of the animal.

Functional redundancy within the *Dlx* enhancer sequences may have emerged as a survival mechanism throughout evolutionary processes.

5.2 The *Dlx* Genes are Regulated Through DLX Proteins Associated with Enhancer Sharing.

The *Dlx* genes expressed in the forebrain are organized into sets of bigene clusters that convergently transcribe towards each other. Two genes in a bigene cluster exhibit overlapping domains of expression in the forebrain, suggesting both redundant and distinct functions (Zerucha and Ekker 2000). The regulatory mechanism governing *Dlx* expression has only recently been studied and there are still many unanswered questions. However, due to the temporal expression patterns found for the *Dlx* genes, the presence of a regulatory cascade has been strongly suggested. The temporal sequence of expression begins with *Dlx2*, followed by *Dlx1, Dlx5* and lastly *Dlx6* (Eisenstat et al. 1999).

In recent years, growing evidence has shown DLX proteins to target the *Dlx* genes themselves through auto or cross-regulatory mechanisms. Additionally, the highly conserved enhancers within the intergenic regions of bigene clusters are known to play a role in *Dlx* regulation and may act alone or with other enhancers to regulate these genes.

In quantifying *Dlx* expression changes in ΔI56i mutants, I have provided evidence for the possibility that *Dlx* gene expression may be regulated through mediation of DLX proteins that may involve enhancer sharing. The I56i element is located between the *Dlx5/6* loci and has two putative DLX binding sites for both DLX1 and DLX2 (Zhou et al. 2004). In ΔI56i mutant mice there is a drastic decrease (~80%) of *Dlx5/Dlx6* expression in the developing forebrain. Consistent with my results, *Dlx1/Dlx2* mutant mice show a substantial decrease in *Dlx5* and *Dlx6* expression (Zerucha et al. 2000) reinforcing the importance DLX proteins play in the maintenance of their own expression. It is important to note that the decrease in

the expression of these genes may also be a result of the loss of binding sites for other, currently unidentified proteins.

In addition to a decrease in both *Dlx5/Dlx6*, ΔI56i mutant mice show a significant increase in *Dlx1* and stable *Dlx2* expression in the developing forebrain. *Dlx1* and *Dlx2* are thought to play regulatory roles on the *Dlx* genes themselves and are located upstream of *Dlx5* and *Dlx6* in a hypothesized genetic pathway. The increase in *Dlx1* expression may be a result of functional compensation for the down-regulation of *Dlx5* and *Dlx6* expression. Considering the significant increase in *Dlx1* expression in these mutant mice, it would be noteworthy to investigate expression changes for *Mash1*, an upstream regulator of *Dlx1*/*Dlx2* that directly binds to I12b, playing an essential role in forebrain regulation (Ghanem et al. 2007). In ΔI12b mutants, *Mash1* expression increases ~ 10% (Yu 2011) and it would not be surprising if an increase was also seen in the ΔI56i mutants, due to its critical role in the production of essential neuronal precursors necessary for early neurogenesis. Perhaps an increase in *Mash1* expression is a protective mechanism to ensure proper neurogenesis of the forebrain, increasing the individual's chance of survival. It is intriguing that *Dlx2* expression does not follow the same increase as *Dlx1* since the two genes have overlapping patterns of expression and functional redundancy as seen in single *Dlx1*/*Dlx2* mutants (Qiu et al. 1997). An alternative hypothesis is that *Dlx1* is being up-regulated by other elements through URE2, a highly conserved regulatory element physically located upstream of *Dlx1* that overlaps with the activity of I12b (Ghanem et al. 2007).

Interestingly, the absence of I12b and vI56i severely inhibits *Dlx* expression and transcripts of all four *Dlx* genes expressed in the forebrain decrease by ~50%. In I12b mutants, there is a slight increase in *Dlx1* expression and *Dlx5/Dlx6* remain stable (Yu 2011), while in the vI56i mice there is a slight decrease in *Dlx5/Dlx6* (Pelletier 2011). Taken

together, the combined effect of the absence of the I12 and presence of the vI56i was enough to induce a marked decrease in *Dlx1/Dlx2* and *Dlx5/Dlx6* expression. The decrease in expression may imply that all four *Dlx* genes expressed in the forebrain are regulated by a mechanism that may include enhancer sharing. It seems that *Dlx5/Dlx6* expression is largely regulated by the I56i enhancer and in its absence or mutations result in severe reduction in expression.

5.3 The I56i Enhancer is Involved in Maintaining a GABAergic Neuronal Phenotype in the Developing Telencephalon Through Regulation of *Gad1/Gad2*.

The *Dlx* genes have expression patterns that tightly overlap in neurons that express γ-aminobutyric acid (GABA) in the developing forebrain. Similarly, the expression patterns of the DLX transcription factors are essentially identical to those of *Gad1/Gad2*, which are the enzymes necessary to synthesize GABA from glutamate. The temporal and spatial correlation of *Gad* and *Dlx* expression illustrates the important involvement the *Dlx* genes have on *Gad* expression. The influence of these genes in regulating *Gad* was exemplified in gain-of-function assays in which *Dlx2* and *Dlx5* were able to induce *Gad* expression in cortical cells of the developing telencephalon, initiating the phenotype of GABAergic neurons (Stuhmer et al. 2002). In mice that lack function of both *Dlx1* and *Dlx2*, there is a significant decrease in *Gad1* expression and differentiation and migration defects in the majority of GABAergic neurons in the telencephalon (Long et al. 2009; Stuhmer et al. 2002). Furthermore, DLX1, DLX2 and DLX5 proteins can interact with *Gad1* enhancer to activate and regulate transcription (unpublished data, reviewed in Panganiban and Rubenstein 2002).

Consistent with Stuhmer's study which demonstrates the importance of the *Dlx* genes in maintaining *Gad* expression, ΔI56i mutant mice show a significant decrease (~50%) in

both *Gad1/Gad2* expressions in the telencephalon. The DLX1 protein can activate *Gad1* through the Gad enhancer, so it may be that the increase in *Dlx1* expression seen in the ΔI56i mutant mice is due to an overcompensation mechanism that is trying to maintain the GABAergic phenotype. In considering the essential role of *Gad* genes in maintaining a GABAergic neuronal phenotype in the telencephalon, it is reasonable to assume that the absence of the I56i is most likely associated with dysfunctional differentiation and migration patterns of these neurons in the telencephalon.

5.4 The I56i Enhancer is Critical in Regulating the Expression of the Non-Coding RNA *Evf*-2, Which Maintains the GABAergic Neuronal Phenotype in the Forebrain.

The discovery of *Evf*-2 as a transcription-regulating ultraconserved ncRNA (trucRNAs) has prompted an increased interest in studying non-coding RNA that are associated with developmental transcriptional regulation. The I56i enhancer overlaps with a small part of the second exon of *Evf-2* and I56i activity overlaps with *Evf-2* expression in the ventral forebrain (Bond 2009). *Evf-2* can recruit DLX2 proteins to the I56i enhancer by forming a stable complex, regulating *Dlx5/Dlx6* and *Gad2* (Feng et al 2006; Bond et al. 2009). Bond et al. have designed *Evf-2* loss-of-function mice by introducing transcriptional termination sites into the first exon of *Evf-2*. (*Evf-2*$^{TS/TS}$mutants). They inserted transcriptional sites into *Evf*-2 instead of deleting DNA fragments because *Evf-2* overlaps with part of I56i and other regulatory factors.

Similar to results from Bond, the lack of *Evf-2* function in ΔI56i mutants is associated with a ~50% decrease in *Gad2* expression in the forebrain, which is expected since *Evf*-2 recruits DLX proteins to the I56i enhancer which are upstream of a hypothetical regulatory

network of *Gad1* and *Gad2*. However, the decrease in *Gad2* expression could solely be a result from decreased *Dlx5/Dlx6* expression in the ΔI56i mutant mice. Finding a reduction in *Gad* expression, the enzyme responsible in synthesizing GABA strongly suggests that *Evf*-2 plays a role in maintaining the production of GABAergic neurons, through regulation of *Gad* expression.

Contradictory to my results, Bond *et al* has shown that the loss of *Evf*-2 function in mice results in a significant increase of *Dlx5* and *Dlx6* expression, suggesting *Evf*-2 has a negative transcriptional role *in vivo* (Bond et al 2009). Further investigation will be necessary to determine the underlying cause of the differences in levels of *Dlx5/Dlx6* expression in each mouse line. However, we can conclude that the loss of *Evf*-2 function in combination with the loss of I56i activity is not substantial in maintaining *Dlx5* and *Dlx6* function.

5.5 *In situ* Hybridization Confirms Changes in *Dlx*, *Gad1/Gad2* and *Evf-2* Expression in ΔI56i Mutant Mice Forebrain.

Impaired gene expression in the I56i mutants led me to carry out *in situ* hybridization experiments on mutant mouse ventral forebrain tissue. I conducted these experiments to specifically discover the impact the absence of this enhancer has on the expression pattern of the developmental genes involved in forebrain development including *Dlx*, *Gad1/Gad2* and *Evf-2.* Any changes in the levels of expression of the genes assayed are global and uniform across the mouse VZ and SVZ of the ventral telencephalon. The *in situ* hybridization results I have obtained show extremely similar changes in gene expression in relation to the levels quantitatively measured through qRT-PCR and all changes in expression are uniform. The resemblance of my qRT-PCR and *in-situ* hybridization results reinforces the certainty that the I56i enhancer is essential in maintaining *Dlx* expression and in part regulates *Gad1/Gad2*

function. Furthermore, the influence of the I56i enhancer on developmental regulators such as the nc-RNA *Evf*-2 is exemplified in its functional termination in I56i mutant mice.

5.6 The I56i Enhancer is Involved in GABAergic Neuronal Development.

The *Dlx* genes tightly regulate the development of GABAergic interneurons, modulating the proper circuitry in the developing forebrain. Since the ΔI56i mutant mice demonstrate a gross down-regulation in *Dlx5/6* and *Gad1/Gad2* expression, we decided to elucidate the effects of the absence of this enhancer on expression of GABAerigc neuronal markers. GABAergic neuronal markers include the calcium binding proteins parvalbumin and calbindin which are both expressed in the cortex, while calbindin expression is also seen in the hippocampus (Potter et al. 2009). I chose to stain the somatosensory cortex of the mutants at P35 with the neuronal markers at a time point in which all neuronal circuits are fully developed and GABAergic neurons have migrated to their correct location in the brain. The amount of parvalbumin and calbindin expressing cells in the somatosensory cortex of ΔI56i mutants are indistinguishable from the wildtype mice. I also stained the somatosensory cortex with an antibody against GABA and saw a reduction in expression in the ΔI56i mutants. Similar to the I56i mutants, *Dlx1/Dlx2* null mice have a severe reduction in both *Dlx5* and *Dlx6* and a major decrease of GABAergic neuronal progenitors (Anderson et al. 1997a). The mechanisms underlying how the *Dlx* genes regulate the development of the GABAergic neuronal subtypes are still yet to be uncovered.

5.7 The Absence of the I56i Enhancer Produces Symptoms Associated with Neurological Disorders in Mice.

As previously discussed, the *Dlx* genes are imperative in regulating the migration and development of GABAergic neurons that are differentiated in the telencephalon (Anderson et

al. 1997). GABA-releasing neurons are inhibitory and in conjunction with excitatory glutamate-releasing neurons, maintain a balance in the neuronal circuitry of the brain. Studies have suggested that any imbalance in GABAergic circuitry may result in an increased excitatory state, leading to neuropsychiatric diseases such as Rett syndrome, autism and anxiety (Acosta and Pearl 2003).

This study has shown that mice lacking the I56i enhancer have a decreased level of *Dlx5/Dlx6* expression which is associated with a corresponding decrease in *Gad1/Gad2* expression in the forebrain. The down regulation of these genes is related to a reduced number of GABAergic cells in the somatosensory cortex, prompting us to investigate the consequences of a disrupted GABAergic network in ΔI56i mutant mice. In doing so, we conducted a series of behavioral tests that measured changes in anxiety, learning and memory which hare all general symptoms of neuropsychiatric disorders (Acosta and Pearl 2003).

I have shown that the absence of the I56i enhancer in mice is associated with impaired fear condition, suggesting learning deficiencies. There is also the possibility that the mutants may have an increase in anxiety however, further investigation is required to determine the strength of this phenotype.

The ΔI56i mutants display a significantly decreased amount of time spent in the small center of a brightly lit open field box, suggesting they have an increase in hyperactivity and anxiety. However, when the mutants underwent both the beam break and elevated plus maze, there was no significant change in activity or amount of time the mutants explored the open areas, indicating these mice did not have an increase in anxiety, as measured by the tests. While results from the beam break and elevated plus maze were not significant, they both had trends towards significance $p<0.06$ and $p<0.077$ respectively. The discrepancy in results

may be related to the basis that the mice were subjected to the beam break test for only two hours during the late afternoon. It may be beneficial to repeat this test and leave the mice in the testing apparatus for 12 hours over night, the time day they are naturally more active. Also, if the anxiety phenotype of the I56i mutant mice as determined by this test is not profound it may be difficult to detect over such a short duration of time and with the sample number I used.

Fear conditioning measures learning and memory through association with predicting aversive events. In mammals, fear conditioning related to learning is a highly complex system regulated in a part by the

amygdala and hippocampal complex. This complex consists of two medial temporal lobe structures that regulate independent memory systems with their own unique functions (Phelps 2004). The hippocampus plays a primary role and is essential in episodic memory, which is the primary memory system in mammals that allows recollection of certain events at individuals will. Amygdala function is involved in more long-term storage of associated emotional events (Phelps 2004). Through the administration of lesions in the amygdala and hippocampus in rats, Phillips and LeDoux have evaluated the role the amygdala and hippocampus play during mouse fear conditioning. The amygdala is involved in fear conditioning responses to simple modality-specific conditioned stimuli and plays an associative role, while the hippocampus is solely involved in memory of more complex polymodal events and plays more of a sensory relay role (Phillips and LeDoux 1992).

During contextual training, which involved placing the mice in the same apparatus that was associated with an aversive shock, the ΔI56i mutant mice had a significant freezing behavior, indicating they remembered the environment they were placed in. The mutant mice

remembered and were aware that the environment they were placed in was associated with an aversive stimulus from the previous day of testing. Because of the significant role the hippocampus plays in memory, we can assume the ΔI56i mutants do not have deficits in hippocampal function. This thought is supported by research confirming the importance of parvalbumin^{+} expressing interneurons in regulating memory (Williams et al. 1992). As previously described in section 5.6, the number of parvalbumin^{+} expressing cells in the ΔI56i mutant forebrain are indistinguishable from wildtypes and may contribute to the functioning memory of the mutants.

While the mutants do not display defects in hippocampal function, results from cue testing provide evidence that the I56i mutants have clear deficits in amygdala function. The mutant mice were unable to associate the tone with the shock and exhibit a much lower inhibition towards the aversive stimulus in comparison to the wildtype mice (Fig 4.14, Cue Tone). Similar to the I56i mutants, mice lacking *Dlx1* function also have reduced fear conditioning inhibitions (Mao et al. 2009). It is interesting that these mice show a decreased response to fear because other mouse models used to analyze inhibitory circuitry show increases in level of fearfulness when given aversive stimuli. For example, mice that have defective $GABA_A$ receptors exhibit enhanced inhibition towards aversive stimuli and heightened responses (Homanics et al. 1997). Perhaps the discrepancy seen within mutant mice with either impaired *Dlx1* function or I56i activity compared to those with defects within GABA receptors is due to receptor mutations affecting the majority of inhibitory synapse, while the Δ*Dlx1* and ΔI56i mutants only have a subset of these synapse affected (Mao et al. 2009). Overall, these results suggest that the absence of the I56i contributes to GABAergic dysfunction which may have implications in neurological disorders.

6 Conclusion

In summary, the results of this study have demonstrated the importance of the *Dlx* *cis*-regulatory elements during varying aspects of forebrain development. We have shown that the *Dlx* genes are regulated in part by enhancer sharing of the highly conserved I12b and I56i enhancers which have functional redundancy, promoting survival of the individual. The down-regulation of *Dlx5/Dlx6* in ΔI56i mutants is associated with a significant decrease in *Gad1/Gad2* expression and loss of function of *Evf*-2. Similar to other studies, the decrease in both *Gad1/Gad2* and *Evf-2* was accompanied by a decrease in number of GABAergic neuronal precursors in the somatosensory cortex, disrupting GABA circuitry in the ΔI56i mutants. The ΔI56i mutant mice have proved an example of how disruptions in GABAergic neuron development can produce a behavioral phenotype leading to increased anxiety and learning deficits, which are both symptoms of neuropsychiatric disorders. Studying highly conserved regulatory elements involved in the *Dlx* regulatory network will not only contribute to an enhanced knowledge of the pathways involved in regulating *Dlx* expression but will shed light on the underlying mechanisms involved in neurological disorders associated with disrupted GABA circuitry.

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Title: Characterization of a distinct subpopulation of striatal projection neurons expressing the*Dlx*genes in the basal ganglia through the activity of the I56ii enhancer

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